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Synergetic effect of interleukin-2 and cellular cytotoxicity against a novel tumor-associated carbohydrate antigen Le^a/Le^a (dimeric Le^a) mediated by monoclonal antibody NCC-ST-421 in adoptive immunization using SCID mice

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Abstract. The murine IgG3 monoclonal antibody NCC-ST-421, raised against a human gastric cancer, shows strong reactivity with dimeric Le^a (Le^a/Le^a; $V^4FucIII^4FucLc_6Cer$) expressed on gastrointestinal cancer cells. ST-421 reacted minimally with non-dimeric or simple Le^a expressed on normal tissues. ST-421 is capable of mediating both antibody-dependent cellular cytotoxicity (ADCC) with human peripheral blood lymphocytes, and complement-dependent cytotoxicity with human complement. Interleukin-2 (IL-2) modulates the function of immunocytes, in particular inducing lymphokine-activated killer (LAK) cell activity and enhancing ADCC. We therefore employed combination immunotherapy with IL-2, LAK, and ST-421-induced ADCC in vitro and in mice with severe combined immunodeficiency (SCID), using target tumor cells expressing Le^a/Le^a antigen. ADCC against human colon cancer cell lines in vitro was enhanced three to four times after preincubation with IL-2. Addition of IL-2 reduced the amount of ST-421 required for efficient ADCC 10- to 100-fold. ADCC was activated by IL-2 earlier (1 day) than the generation of LAK cells (3–4 days), and at lower concentration of IL-2. These effects were specific for ST-421, as demonstrated by experiments with irrelevant antibody or irrelevant target cells. An anti-(Fc receptor) antibody blocked the ADCC but not the LAK activity in vitro. The enhancement of ADCC by IL-2 may be caused by activation of effector cells expressing Fc receptors. In vivo experiments using SCID mice inoculated with human colon cancer showed a significant tumor-growth-suppressive effect after combined therapy using human peripheral blood lymphocytes, LAK, IL-2, and ST-421. In summary, adoptive immunization with human lymphocytes activated by IL-2 and ST-421 effectively suppressed growth of gastrointestinal cancer cells expressing Le^a/Le^a.

Key words: Interleukin-2 – Extended α1→4-fucosylated type-1 chain antigen – Lymphokine-activated killer cell – Antibody-dependent cytotoxicity.

Introduction

Many monoclonal antibodies (mAb) have been generated against various kinds of cancer-associated antigens. The anticancer activity of some of these mAb has been established in patients with lymphoma, melanoma, neuroblastoma, and colon cancer [2, 8, 9, 12, 19]. These particular mAb are capable of mediating antibody-dependent cellular cytotoxicity (ADCC) with human peripheral blood lymphocytes (PBL) in vitro. We have shown previously that murine IgG3 mAb NCC-ST-421 [21] is directed against cancer-associated novel repetitive Le^a or Le^a/Le^a, but not non-dimeric or simple Le^a antigens, which are present in normal tissues (Table 1). ST-421 induces strong ADCC with human PBL, and complement-dependent cytotoxicity (CDC) with human complement, and strongly suppresses growth of Le^a/Le^a-expressing target tumor cells in vitro and in vivo. However, the antibody did not produce any inflammatory or histological changes in normal tissues expressing non-dimeric or simple Le^a but not Le^a/Le^a [22].

Recent work has shown that PBL activated with interleukin-2 (IL-2) in vitro or in vivo are able to lyse a wide variety of fresh or cultured tumor cells, but are generally ineffective against normal cells or xenogenic tumor cells [5]. Therapy of human cancers with lymphokine-activated

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Table 1. Reactivities of mAb ST-421, directed to a novel repetitive Le^a/Le^a epitope

Antigen	Presence in: Tumor cells and tissues	Presence in: Normal tissue	Reactivity with ST-421[a]	Structure
Dimeric Le^a (Le^a/Le^a) ($V^4III^4Fuc_2Lc_6$)	+	–	+++	Galβ1→3GlcNAcβ1→3Galβ1→3GlcNAcβ1→3Galβ1→4Glc→Cer 4 4 ↑ ↑ Fucα1 Fucα1
Extended Le^a on type-2 chain	–	+	+	Galβ1→3GlcNAcβ1→3Galβ1→4GlcNAcβ1→3Galβ1→4Glc→Cer 4 ↑ Fucα1
Extended Le^a on type-1 chain	–	–	+	Galβ1→3GlcNAcβ1→3Galβ1→3GlcNAcβ1→3Galβ1→4Glc→Cer 4 ↑ Fucα1
Simple Le^a (lactofucopentaose II Cer)	++	++	+	Galβ1→3GlcNAcβ1→3Galβ1→4Glc→Cer 4 ↑ Fucα1

[a] Determined by antibody binding to solid-phase antigen on plastic plates and on thin-layer chromatography, and by immunohistological staining

killer (LAK) cells generated in vitro and IL-2, or IL-2 alone, may occasionally induce tumor regression [18]. However, this therapy appears to be effective for relatively few tumor types and is often associated with severe toxicity. No case has been reported so far in which the target antigen is chemically well-defined as a blood-group-related carbohydrate antigen.

On the other hand, some studies have shown an increase in ADCC against cultured melanoma and neuroblastoma cells when effector cells are preincubated with IL-2 [8, 14]. PBL activated with IL-2 (LAK) are heterogeneous cell populations and include cells of the natural killer (NK) phenotype. It has also been shown that murine IgG3 interacts with large granular lymphocytes, the activity of which can be enhanced by IL-2.

Transfer of a functional human immune system to mice with severe combined immunodeficiency (SCID) has been reported recently [1, 11, 13]. Two approaches have been used. In the first approach, $(1–10) \times 10^7$ human PBL were i.p. injected into SCID mice. These mice did not develop lethal graft-versus-host disease, and produced human immunoglobulins [13]. In the second approach, SCID mice were grafted with human embryonal thymus and lymph nodes. This allowed differentiation of human T cells following i.v. injection of human bone marrow cells [11]. These findings suggest that SCID mice reconstituted with human lymphoid cells may be used as a model of the human immune system.

In the present study, mAb therapy combined with adoptive cell transfer treatment with LAK cells and IL-2, using Le^a/Le^a-expressing tumor cells as a target, gave encouraging results. To evaluate the possible benefits of this combined therapy in the treatment of gastrointestinal tumors, we analyzed the effects of IL-2 and ST-421 on ADCC mediated by adopted human effector cells against human colon cancer cell growth in SCID mice.

Materials and methods

Antibodies. NCC-ST-421 is an IgG3 murine mAb recognizing Le^a/Le^a, a novel antigen with an extended type-1 chain ($V^4FucIII^4FucLc_6Cer$; see Table 1) [20, 22]. This mAb, which can mediate ADCC and CDC, was purified by ion-exchange chromatography. J30 (IgG3) directed to CD9 and J3-119 (IgG1) directed to CD19, kindly provided by Dr. J. M. Pesando, were used as negative controls [15, 16]. 3G8 (AMAC Inc., Westbrook, Me.), an IgG1 murine mAb directed to FcrRIII (CD16) receptor, was used for the blocking assay [3].

Cell lines. Colo205 (human colon cancer) was obtained from the American Type Culture Collection, Bethesda, Md. Lu65 (human lung large-cell carcinoma) was kindly provided by Dr. Setsuo Hirohashi, National Cancer Center Research Institute, Tokyo, Japan [23]. Colo205 is positive for ST-421-reactive antigen, whereas Lu65 is negative by immunohistochemical analysis. Both cell lines were grown in RPMI-1640 medium supplemented with 10% heat-inactivated bovine serum albumin (Hyclone, Logan, Utah) and 2 mM glutamine.

Effector cells. Human PBL were obtained from healthy volunteer donors. Heparinized whole blood was fractionated by density-gradient centrifugation on Ficoll/Hypaque (Pharmacia, Piscataway, N.J.), and the mononuclear cell fraction washed three times with RPMI medium. Monocytes were depleted by adhesion to plastic tissue culture flasks (Falcon, Lincoln Park, N.J.) for 1 h at 37° C in RPMI medium with 10% fetal bovine serum (FBS). Non-adherent cells were used as effector cells.

IL-2 activation of effector cells. PBL (2×10^4/ml) were cultured in RPMI medium with 10% FBS and 2 mM L-glutamine at 37° C in 5% CO_2. Various concentrations of recombinant IL-2 (Boehringer Mannheim, Indianapolis, Ind.) were added as described in Results, and the effector cells were incubated for various lengths of time. The specific activity of IL-2 was more than 2.0×10^6 units/mg protein.

Chromium release assay. Target cells (2×10^6) were labeled with 200 μCi sodium [^{51}Cr]chromate (25 mCi/ml; Amersham, Arlington Heights, Ill.) in 200 μl phosphate-buffered saline (PBS) for 1 h, washed twice, and incubated in RPMI medium with 10% FBS at 37° C in 5% CO_2 for 1.5 h. Effector cells were added to 2×10^4 target cells in 96-well round-bottom plates (Falcon) in a final volume of 200 μl. Cytotoxicity was measured in

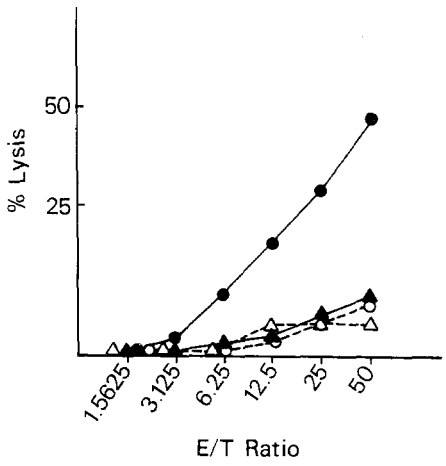

Fig. 1. Antibody-dependent-cellular-cytotoxicity(ADCC)-mediated by ST-421 against Colo205 and Lu65 cells in vitro, using peripheral blood lymphocytes (PBL) as effector cells. ADCC was measured using either ST-421 against Colo205 (●) and Lu65 (○), or mAb J30B against Colo205 (▲) and Lu65 (△) with human PBL at various E:T ratios. Both mAb were used at 2.5 μg/ml. Points represent means of triplicate experiments, expressed relative to the mean ±SD percentage inhibition of control

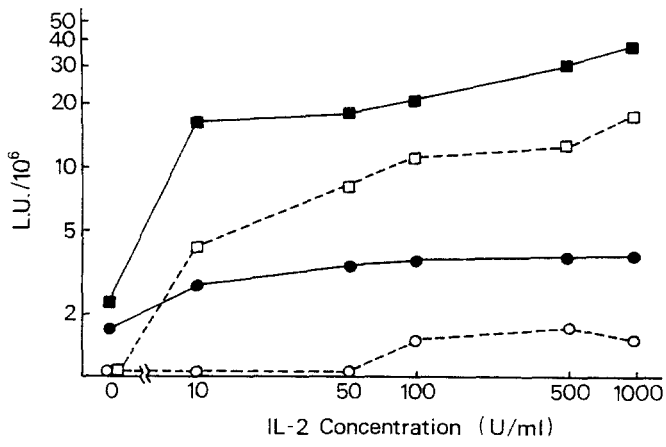

Fig. 2. Effect of interleukin-2 (IL-2) concentration on ADCC and lymphokine-activated killer (LAK) activity. Normal human PBL were incubated with various concentrations of IL-2 for 24 h or 72 h. Cytotoxicity against Colo205 was measured with or without ST-421 at 2.5 μg/ml; the E:T ratio was 25:1. 24-h incubation: ●, ADCC; ○, LAK; 72-h incubation: ■, ADCC; □, LAK. *Ordinate*: 1 LU represents the effector cell number that destroys 30% of 10^4 cells, based on percentage lysis and E:T ratios. The unit is expressed per 10^6 cells by the equation shown in the text. Points represent means of triplicate experiments. Cytotoxicity (LU) as shown in Figs. 2–5 is calculated on the basis of the mean ±SD percentage inhibition of the control; therefore SD values of these units cannot be expressed mathematically, and no SD bars are shown

the presence or absence of ST-421 at the concentrations required by the experimental design, as described previously [22]. Plates were incubated for 4 h at 37° C, centrifuged at 100 rpm for 2 min, and 100 μl supernatant was removed for gamma counting. Maximum isotope release was measured by incubation of target cells with 5% Triton X-100. Spontaneous release was determined by incubation of the ^{51}Cr-loaded target cells with medium alone. The percentage specific lysis was calculated as:

$$\text{lysis (\%)} = \frac{\text{experimental release} - \text{spontaneous release}}{\text{maximum release} - \text{spontaneous release}} \times 100$$

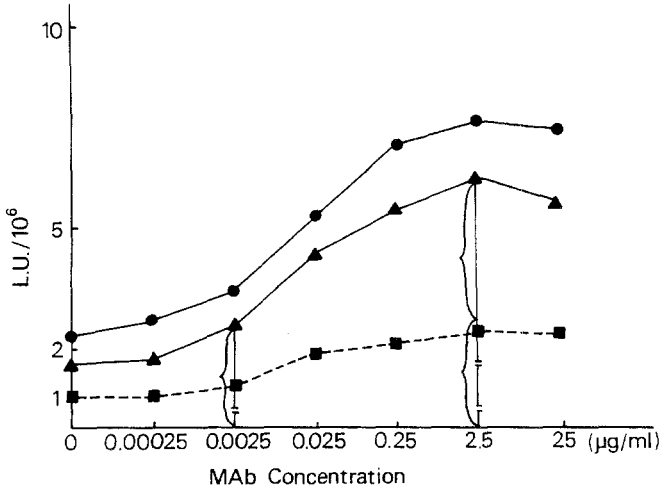

Fig. 3. Effect of mAb concentration on ADCC with IL-2-activated PBL. Cytotoxicity against Colo205 was measured with various concentrations of ST-421 using human PBL preincubated for 48 h with or without IL-2. The E:T ratio was 25:1. IL-2 concentrations: ■, 0 U/ml; ▲, 10 U/ml; ●, 100 U/ml. Points represent means of triplicate experiments

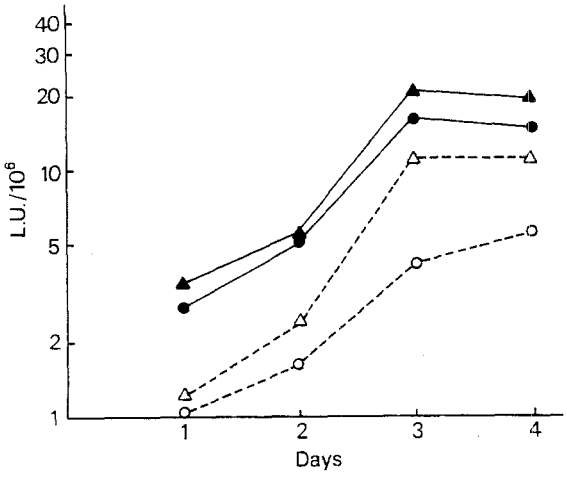

Fig. 4. Effect of IL-2 incubation time on ADCC and LAK activity. Normal human PBL were incubated for 1–4 days with IL-2 at 10 U/ml or 100 U/ml. Cytotoxicity against Colo205 was measured with or without ST-421 at 2.5 μg/ml. The E:T ratio was 25:1. At 10 U/ml: ●, ADCC; ○, LAK. At 100 U/ml: ▲, ADCC; △, LAK. Points represent means of triplicate experiments

Data are shown as either the mean percentage lysis or as lytic units (LU) per 10^6 cells; 1 LU was defined as the number of mononuclear cells required to obtain 30% specific lysis, according to the method of Pross et al. [17]; i.e., cytotoxicity (LU)/10^6 cells = $k\,e^{y \cdot \ln x}$, where $k = 10^6/10^4 = 10^2$, y = (% lysis – 30%)/slope, and x = E/T ratio at which the observed percentage lysis occurred. "Slope" refers to an appropriate value (12.0) obtained from the killing curve (lysis versus ln E:T ratio) (not shown).

Evaluation of antitumor effects in vivo. Homozygous CB-17 scid/scid mice (SCID) were maintained and bred under specific- pathogen-free conditions. Animals 6–8 weeks old (10–15 g) were used for experiments. Colo205 cells (1×10^7) were inoculated s.c. into the backs of animals. Tumors were measured (length and width), and tumor weight (mg) was estimated by the formula [length (mm) × width^2 (mm^2)]/2. The relative mean tumor weight was calculated as m_i/m_0, where m_i is mean tumor

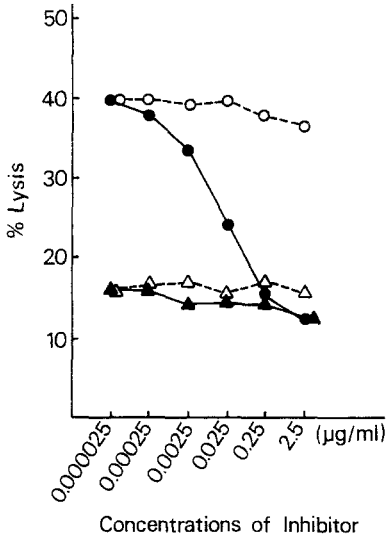

Fig. 5. Effects of anti-(Fc receptor) mAb 3F8 on ADCC and LAK activity. Normal human PBL were incubated for 4 days with IL-2 at 10 U/ml. ADCC by ST-421 at 2.5 μg/ml and LAK activity against Colo205 were measured with mAb 3G8 and J3-119 at various concentrations. E:T ratio was 25:1. ●, ADCC with 3G8; ○, ADCC with J3-119; ▲, LAK with 3G8; △, LAK with J3-119. Points represent means of triplicate experiments

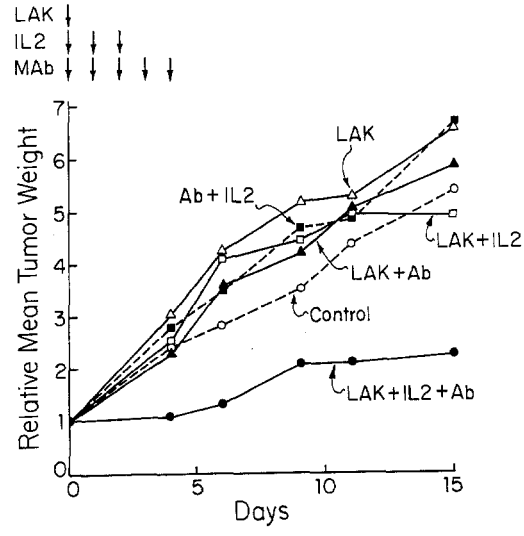

Fig. 6. Antitumor effects of ST-421 and IL-2 in vivo. Mice with severe combined immunodeficiency (SCID) developed s.c. tumors 10 days after inoculation of 10^7 Colo205 cells s.c. on day 0. IL-2 was given as 10 000 U i.p. twice a day from day 0 to day 3. ST-421 (100 μg) was given i.p. from day 0 to day 5. 10^7 PBL pre-activated with IL-2 (10 U/ml) for 3 days were administered i.v. on day 0. Results from one experiment are shown here. Replicate experiments gave similar results. Each point represents a mean value of "relative mean tumor weight" (m_i/m_0) (see Materials and methods). Because of the nature of this parameter, SD values cannot be calculated or shown

weight at a given time and m_0 is mean tumor weight at time of initial treatment (approximately 40 mg). Treatment with IL-2, ST-421, or PBL activated with IL-2 for 3 days at 10 U/ml (LAK) was started when the estimated tumor weight reached approximately 40 mg (10 days after tumor inoculation). ST-421 (100 μg in 0.2 ml PBS) was given i.p. once a day for the first 5 days, and IL-2 (10000 units) was given i.p. twice a day for the first 3 days. LAK cells (10^7) were given i.v. on day 0. Different experimental groups i–vi received the following: (i) no treatment, (ii) ST-421 plus IL-2, (iii) LAK alone, (iv) ST-421 plus LAK, (v) LAK plus IL-2, or (vi) ST-421 plus IL-2 plus LAK.

Because of the nature of the relative mean tumor weight, a simple mean value was taken for each group and the SD cannot be shown. However, tumor weight ±SD was calculated by measuring the actual tumor weight following extirpation of the tumor 15 days after initiation of treatment. The statistical significance of the difference between the control group (group a) and each experimental group was determined by Student's *t*-test.

Results

ADCC using ST-421

Previous experiments have shown that ST-421 mediates ADCC against various cell lines that express Le^a/Le^a. We observed that ST-421 mediated ADCC against Le^a/Le^a-positive Colo205 cells but not against Le^a/Le^a-negative Lu65 cells (Fig. 1). ADCC was correlated with the E:T ratio. Effector cells were fresh PBL from healthy volunteer donors, and the mAb was purified by ion-exchange chromatography. The Colo205 cell line was used in subsequent experiments because it was resistant to NK activity and gave consistent results for ADCC.

ADCC with IL-2-activated effector cells

We observed the pharmacokinetics of IL-2 enhancement of ADCC by stimulating PBL with IL-2 at various concentrations. Figure 2 shows the dose/response curve after 24 h and 72 h IL-2 incubation. ADCC was enhanced by IL-2 at a concentration of 10 U/ml after a 1-day incubation, whereas LAK activity was weakly induced by IL-2 at 100 U/ml after 1 day. After preincubation with IL-2 at 1000 U/ml for 1 day, ADCC was twice as high as LAK activity. During 72 h incubation, LAK activity and ADCC increased in a dose-dependent fashion. At low concentrations of IL-2 (10 U/ml), a four-fold increase in ADCC relative to LAK activity (4.5–19 LU) was induced. However, higher IL-2 concentrations (10–1000 U/ml) produced only small increases in ADCC activity (20–40 LU).

PBL and IL-2-activated PBL (from the same donor) were tested at a fixed E:T ratio (25:1), using mAb concentrations ranging from 0 to 25 μg/ml (Fig. 3). For both IL-2-activated and non-activated PBL, the concentration of mAb required for maximal lysis was 2.5 μg/ml, and that required for 50% maximal lysis was near 0.0025 μg/ml. The ADCC curves were similar in shape for activated and non-activated PBL, but differed in magnitude; ADCC was always higher for activated PBL at any given mAb concentration (Fig. 3).

PBL were stimulated with 10 U/ml or 100 U/ml IL-2 for various lengths of time and tested for ADCC in comparison with LAK activity (Fig. 4). The relative contribution of mAb to lysis of target cells was high during the first 3 days, and maximal lytic activity was noted at 3 days. Although ADCC began to decline after 3 days at both IL-2 concen-

trations, LAK activity was maximal between days 3 and 5. ADCC at 10 U/ml was similar to that at 100 U/ml IL-2.

Effects of murine mAb 3G8 (directed to Fc receptor) on ADCC and LAK activity were studied in vitro. PBL activated by IL-2 at 10 U/ml for 4 days were used as effector cells in ADCC. ADCC was blocked by 3G8 in a dose-dependent fashion (Fig. 5). In contrast, LAK activity at 4 days was unaffected by 3G8. Neither ADCC nor LAK activity was affected by mAb J3-119, which is of the same subclass (IgG1) as 3G8. ADCC using fresh PBL as effector cells was also blocked by 3G8 (data not shown). Cytotoxicity (LU) as shown in Figs. 2–5 is calculated on the basis of the mean ±SD percentage inhibition of the control; therefore SD values of these units cannot be expressed mathematically, and no SD bars are shown.

Tumor growth suppression in vivo by adoptive immunization using human PBL, human LAK, IL-2, and mAb ST-421

In in vitro experiments, growth of Le^a/Le^a-positive Colo205 cells (but not Le^a/Le^a-negative Lu65 cells) was inhibited by ADCC induced by IL-2-activated human effector cells or ADCC induced directly by ST-421 (see above). We therefore examined the effects of adoptive immunization using various combinations of human PBL, human LAK, IL-2, and ST-421 on Colo205 cell growth in SCID mice. Colo205 cells were transplanted into 30 mice, which were divided into six experimental groups of 5 animals each. Colo205 growth was significantly reduced by infusion of LAK and IL-2 in the presence of ST-421 within 15 days. However, other treatment combinations (e.g., IL-2 plus ST-421; LAK plus ST-421; LAK plus IL-2), in which one of these three components was omitted, had little or no effect on Colo205 growth within the same period (Fig. 6). Replicate experiments with the same design and same number of mice gave consistently similar results. Each point shown in Fig. 6 represents a mean value of relative mean tumor weights (m_i/m_0; see Materials and methods); therefore SD cannot be calculated. Actual tumor weights were measured and the statistical significance of differences in mean tumor weight ±SD, determined by Student's *t*-test for control Group i versus experimental groups. The mean tumor weight for group vi was different (smaller) from that for group i at the $P<0.01$ level. Group v was different from group i at the $0.05<P<0.1$ level (insignificant). For groups ii, iii, and iv, the mean tumor weight was higher than that for group i.

Discussion

mAb that are directed to cancer-associated antigens and mediate ADCC with human effector cells have obvious potential utility in cancer immunotherapy. Some mAb capable of mediating ADCC have demonstrated clinical utility in therapy of patients with melanoma or neuroblastoma [2, 9]. Although the mechanism of tumor regression in these patients is unclear, tumor cell killing by ADCC or CDC may be important. mAb ST-421 is directed mainly to a novel difucosylated antigen with an extended type-1 chain, termed dimeric Le^a or Le^a/Le^a. It is only weakly reactive with simple Le^a or non-repetitive extended Le^a antigens present in normal tissues (Table 1). ST-421 is able to mediate ADCC with human PBL and CDC with human complement in vitro, and to inhibit tumor growth in vivo, and is therefore a potential agent for immunotherapy [22].

The present study clearly demonstrates (a) enhancement of ST-421-mediated ADCC by two- to fourfold in vitro after incubation with IL-2 and (b) a synergetic effect of IL-2 and ADCC directed against a novel, chemically well-defined structure. ADCC with PBL from eight different donors was, without exception, enhanced by IL-2. At low doses (10–50 U/ml) of IL-2, ADCC was enhanced and responded more rapidly to stimulation than did LAK activity. PBL activated by IL-2 were able to maintain similar or higher levels of ADCC compared to PBL without IL-2, using 10 to 100 times less mAb.

Munn et al. previously reported enhancement of ADCC by IL-2 in vitro using anti-GD2 mAb. Maximal ADCC was reached 1 day after activation with 1000 U/ml IL-2 [14]. On the other hand, Gill et al. reported that maximal ADCC and LAK were reached after a 3-day incubation with this same concentration of IL-2 [4]. We observed that both ADCC and LAK activity reached maxima after 3 days of activation with IL-2 at 1000 U/ml. The optimal duration of IL-2 incubation may vary with the mAb used. Although 1000 U/ml IL-2 was needed to obtain the highest lytic activity in ADCC after 3 days of activation, ADCC at 1000 U/ml IL-2 was almost the same as that at 10 U/ml after 1 day of activation.

It has been reported that LAK cell precursors express the Fc receptor [4], and LAK cells themselves can have either an Fc-receptor-positive or -negative phenotype during activation with IL-2 [14]. However, IL-2-enhanced ADCC is completely abolished by addition of anti-Fc receptor antibody. LAK is not affected by this treatment. The cause of enhancement may be that IL-2 induces not only expression of Fc receptor, but also proliferation of cells that express the receptor.

In vivo tumor growth in nude mice was significantly reduced by treatment with ST-421 plus IL-2 plus LAK, or IL-2 plus LAK, but not by treatment with ST-421 plus LAK, ST-421 plus IL-2, or LAK alone. SCID mice were used as a model for human effector cell function in ADCC. Human NK cells were not found in SCID mice reconstituted with human PBL (data not shown). SCID mice are known to have precursors of LAK cells that can be activated by IL-2 [13]. ST-421 was found to stimulate in vitro ADCC of SCID mouse spleen cells and human PBL. It is possible that adoptively transferred LAK cells were activated by IL-2, and that some of them could act as effector cells in ADCC.

Since gastrointestinal tumors are resistant to conventional therapies, new treatments are needed. Immunotherapy using various mAb has been attempted on patients with melanoma, neuroblastoma, and lymphoma. However, only one mAb (17-1A) has been applied clinically in patients with gastrointestinal cancer. Phase II clinical trials using 17-1A indicate little efficacy and minimal side-effects

[19]. A major limitation may be the inadequacy of host effector responses to the therapeutic mAb. Our preliminary results with ADCC and IL-2 are encouraging for future clinical studies in patients with gastrointestinal cancer. Serum IL-2 levels in the 5–10 U/ml range are probably attainable by a constant infusion of IL-2 [10]. Serum antibody concentrations of 10–20 ng/ml are attainable by i.v. infusion. There are reports of enhanced ADCC following in vivo therapy with IL-2 [6, 7]. The combination of ST-421 and IL-2 may prove to be valuable in therapy of gastrointestinal cancer.

Since the antigen is chemically well-defined, and its chemical synthesis is possible, we envision production of a large number of mAb with better affinity and cooperativity with IL-2 which will be useful in combination therapy for suppression of gastrointestinal cancers expressing this novel antigen. It should be noted that simple Le^a or non-repetitive Le^a antigens expressed on normal tissues are not the targets of ST-421, nor of a combination of ST-421-induced ADCC and IL-2.

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