

Activating Fc Receptors Are Required for Antitumor Efficacy of the Antibodies Directed toward CD25 in a Murine Model of Adult T-Cell Leukemia

Meili Zhang,¹ Zhuo Zhang,¹ Kayhan Garmestani,¹ Carolyn K. Goldman,¹ Jeffrey V. Ravetch,⁴ Martin W. Brechbiel,² Jorge A. Carrasquillo,³ and Thomas A. Waldmann¹

¹Metabolism Branch and ²Radiation Oncology Branch, Center for Cancer Research, National Cancer Institute, Bethesda, Maryland; ³Nuclear Medicine Department, Clinical Center, National Institutes of Health, Bethesda, Maryland; and ⁴Laboratory of Molecular Genetics and Immunology, The Rockefeller University, New York, New York

ABSTRACT

We previously showed therapeutic efficacy of humanized anti-Tac (HAT), murine anti-Tac (MAT), and 7G7/B6 monoclonal antibodies, which recognize CD25, for human adult T-cell leukemia (ATL) in a murine model. In this study, we investigated the mechanism underlying the tumor-killing action mediated by these antibodies on an ATL model in nonobese diabetic/severe combined immunodeficient (SCID/NOD) wild-type mice that lack effective T and natural killer (NK) cells and in SCID/NOD Fc receptor common γ chain knockout (FcR $\gamma^{-/-}$) mice. The ATL model was established by i.p. injection of human ATL cells (MET-1) into SCID/NOD wild-type or SCID/NOD FcR $\gamma^{-/-}$ mice. HAT, MAT, and 7G7/B6 were given to the leukemia-bearing mice at a dose of 100 μ g weekly for 4 weeks. The three antibodies inhibited the leukemia growth significantly in SCID/NOD wild-type mice, as monitored by serum levels of human β 2-microglobulin ($P < 0.01$), and prolonged survival of the leukemia-bearing SCID/NOD wild-type mice ($P < 0.01$) as compared with the control group. However, none of the antibodies manifested efficacy on the leukemia growth and survival of the SCID/NOD FcR $\gamma^{-/-}$ mice bearing MET-1 leukemia. In a pharmacokinetics study, the blood concentrations of the radiolabeled antibodies decreased with time similarly in SCID/NOD wild-type and SCID/NOD FcR $\gamma^{-/-}$ mice. Although NK cells may play a role in humans, in this murine model FcR γ receptors on non-NK cells, such as polymorphonuclear leukocytes or monocytes, are required for the tumor-killing action of the antibodies directed toward CD25.

INTRODUCTION

Adult T-cell leukemia (ATL) develops in a small proportion of human T-cell lymphotropic virus I-infected individuals (1). The leukemia consists of an overabundance of malignant activated T cells, which are characterized by the expression of CD25, or interleukin (IL)-2R α , on their cell surfaces (2–4). There presently is no accepted curative therapy for ATL, and patients progress to death with a median survival duration of 9 months for those with acute ATL and 24 months for those with chronic ATL (1).

The observation that IL-2R α is not expressed by normal resting cells, but is expressed by ATL and some other malignant cells, provided the rationale for the use of monoclonal antibodies directed toward IL-2R α (5). A preclinical *in vivo* murine model of ATL was developed by introducing leukemic cells (MET-1) from an ATL patient into nonobese diabetic/severe combined immunodeficient (SCID/NOD) mice that lack effective T and natural killer (NK) cells (6), and new therapeutic approaches have been tested in this model before initiating clinical trials (6–11). In initial studies, antibodies to IL-2R α , including humanized anti-Tac (HAT), murine anti-Tac (MAT), and 7G7/B6, inhibited the progression of the leukemia and prolonged the survival of the leukemia-bearing mice (6). Furthermore, some partial and rare complete remissions were obtained in patients

with ATL treated in clinical trials with HAT, MAT, and these intact antibodies armed with ⁹⁰Y used in an effort to develop yet more effective IL-2R α -directed agents (12, 13). However, the mechanism underlying the tumor-killing action mediated by the unmodified antibodies directed toward CD25 remains unclear. Several mechanisms theoretically could be involved in the action of anti-IL-2R α in the MET-1 ATL model. These include the blockade of IL-2 interaction with its growth factor receptor IL-2R α with consequent cytokine deprivation mediated cell death, complement-dependent cytotoxicity, and antibody-dependent cellular cytotoxicity. Because there were no IL-2 mRNA expression and IL-2 production by the MET-1 leukemic cells (6) and because 7G7/B6 did not block the IL-2 binding site on the IL-2R α (14), the prevailing view of a sole mechanism for anti-IL-2R α action that is the blockade of the interaction of IL-2 with its receptor was not supported. We also have presented evidence that excluded complement-dependent cytotoxicity (6). Classical antibody-dependent cellular cytotoxicity mediated by NK cells that may be important in immunologically intact humans also does not appear to be a likely mode of action in this model because the SCID/NOD mice used as the recipients of the ATL in our study virtually lacked functional NK cells. Nevertheless, we showed that the F(ab')₂ fragment of HAT in contrast to intact antibody was not effective in this model (6). Because the F(ab')₂ fragment of HAT lacks the Fc segment that is required for complement-dependent cytotoxicity and other Fc-mediated cytotoxicity, we considered the possibility that Fc-dependent antibody-mediated cytotoxicity involving FcR γ I- or FcR γ III-expressing cells, such as monocytes or polymorphonuclear leukocytes, was a potential mechanism of action of these anti-CD25 antibodies in this model and, by inference, that FcR γ III-mediated killing might play a role in the therapeutic efficacy observed in the IL-2-independent phase of ATL patients. To address this hypothesis, we evaluated the therapeutic efficacy of HAT, MAT, and 7G7/B6 in our ATL model in SCID/NOD wild-type and SCID/NOD Fc receptor common γ chain knockout (FcR $\gamma^{-/-}$) mice. We showed that the three antibodies evaluated in this study had therapeutic efficacy on the MET-1 leukemia in SCID/NOD wild-type mice, whereas this efficacy was lost in SCID/NOD FcR $\gamma^{-/-}$ mice, indicating that the expression of the Fc receptors FcR γ I and FcR γ III that involve the Fc γ chain is required for the effective action of the antibodies directed toward CD25 *in vivo*.

MATERIALS AND METHODS

Monoclonal Antibodies. The HAT antibody (daclizumab) was obtained from Hoffmann-La Roche (Nutley, NJ). MAT was produced as described previously (2, 15) by fusion of NS-1 mouse myeloma cells with spleen cells of mice that had been immunized with a cell line derived from a patient with ATL. Large quantities of the antibody were produced by inoculation of hybridoma cells into the peritoneal cavity of BALB/c mice and then purifying the mouse IgG2a anti-Tac from the resulting ascites using DEAE cellulose chromatography. HAT and MAT recognize IL-2R α . 7G7/B6 is a mouse IgG2a directed toward an epitope of the IL-2R α peptide other than that identified by anti-Tac (14). The 7G7/B6 was purified from supernatants of a hybridoma (American Type Culture Collection, Manassas, VA) using ImmunoPure Protein A columns (Pierce, Rockford, IL).

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Requests for reprints: Thomas A. Waldmann, Metabolism Branch, NCI, NIH, Building 10, Room 4N115, 10 Center Drive, Bethesda, MD 20892-1374. Phone: 301-496-6656; Fax: 301-496-9956; E-mail: tawald@helix.nih.gov.

Mouse Model of ATL. SCID/NOD mice were purchased from The Jackson Laboratory (Bar Harbor, ME), and SCID/NOD FcR $\gamma^{-/-}$ mice were generated in the laboratory of Jeffrey Ravetch (Rockefeller University, New York, NY). The ATL cell population, MET-1, was established from the peripheral blood of a patient with acute ATL, and the leukemic cells were maintained by serial transfer in SCID/NOD mice. MET-1 cells have a distinct phenotype elucidated by fluorescence-activated cell sorter analysis: CD3 dim, CD4 positive/negative, CD7, CD20 negative, and CD25 positive. The ATL model was established by i.p. injection of 1.5×10^7 MET-1 cells into SCID/NOD wild-type or SCID/NOD FcR $\gamma^{-/-}$ mice as described previously (6, 8). The therapy experiment was performed on the ATL-bearing mice when their serum-soluble IL-2R α (sIL-2R α) levels were >1000 pg/ml, which occurs ~ 10 – 14 days after tumor inoculation. All of the animal experiments were performed in accordance with NIH Animal Care and Use Committee guidelines.

Therapy Study. Therapeutic studies were performed in MET-1 leukemia-bearing SCID/NOD wild-type and SCID/NOD FcR $\gamma^{-/-}$ mice. The leukemia-bearing mice were randomly assigned to groups that had comparable levels of the surrogate tumor marker, sIL-2R α (Fig. 1A). Groups of 15 mice were

injected i.v. with 100 μ g of HAT, MAT, or 7G7/B6 or with 200 μ l of PBS weekly for 4 weeks. An additional group received 100 μ g of HAT and 100 μ g of 7G7/B6 weekly for 4 weeks. Throughout the experiment, tumor progression was monitored by serum levels of soluble β 2-microglobulin (β 2 μ), a surrogate tumor marker, and by Kaplan-Meier analysis of the survival of the mice.

Monitoring of Tumor Growth. Measurements of the serum concentrations of the sIL-2R α and/or soluble human β 2 μ were performed using ELISA. β 2 μ , which was used as a surrogate tumor marker, was measured after therapy to monitor the growth of the leukemia. The ELISA kits were purchased from R&D System (soluble Tac, catalogue no. DR2A00; soluble β 2 μ , catalogue no. DBM200; Minneapolis, MN). The ELISAs were performed as indicated in the manufacturer's kit inserts.

Radiolabeling and Radioimmunoactivity of Antibodies. HAT, MAT, and 7G7/B6 were labeled with 125 I at specific activities of 74–111 kBq/ μ g by using the chloramine-T method. Kit225IG3, a leukemic T-cell line that expresses CD25 on the cell surface, was maintained in RPMI 1640 containing 10% heat-inactivated fetal bovine serum, 100 units/ml penicillin, and 100 μ g/ml streptomycin in an atmosphere containing 5% CO $_2$. Immunoreactivity of 125 I-HAT and 125 I-7G7/B6 was evaluated as described previously (16). Briefly, 125 I-HAT or 125 I-7G7/B6 (5 ng/100 μ l) was incubated with an increasing number of Kit225IG3 cells (1×10^4 – 1×10^7) with or without unlabeled HAT or 7G7 (25 μ g/tube) inhibition at 4°C for 1 h. After centrifugation, the supernatant was aspirated, and the radioactivity bound to the cells was counted in a gamma counter.

Pharmacokinetics of HAT, MAT, and 7G7/B6. To define the clearance rate of infused antibodies from the blood in SCID/NOD wild-type and SCID/NOD FcR $\gamma^{-/-}$ mice, 100 μ g (the same dose as that used in the therapeutic study) of 125 I-labeled HAT, MAT, or 7G7 were injected into SCID/NOD wild-type or SCID/NOD FcR $\gamma^{-/-}$ mice ($n = 5$). Serial blood samples were taken at different time points after injection and counted in a gamma counter. To determine the area under the curve (AUC), the percentage of the injected dose per gram of blood (%ID/g) was calculated and integrated using the trapezoidal rule method (GraphPad Prism version 4p; GraphPad Software, San Diego, CA).

Statistical Analysis. The serum levels of β 2 μ at different time points for the different treatment groups and AUCs of each antibody between SCID/NOD wild-type and SCID/NOD FcR $\gamma^{-/-}$ mice groups were analyzed using the Student's t test for unpaired data. Statistical significance of differences in survival of the mice in different treatment groups was determined by the log-rank test using StatView program (Abacus Concepts Inc., Berkeley, CA).

RESULTS

Therapeutic Study with HAT, MAT, and 7G7/B6. The therapeutic efficacy of HAT, MAT, and 7G7/B6 for the management of ATL was shown in MET-1-bearing SCID/NOD wild-type mice but not in MET-1-bearing SCID/NOD FcR $\gamma^{-/-}$ mice. In the therapeutic study, the HAT, MAT, and 7G7/B6 monoclonal antibodies, all of which are directed toward the IL-2R α on the leukemic cells, were injected i.v. weekly for 4 weeks at a dose of 100 μ g in 200 μ l. All of the antibodies manifested therapeutic efficacy in SCID/NOD wild-type mice bearing MET-1 leukemia as shown by their effects on the serum levels of β 2 μ , a surrogate tumor marker that was indicative of the tumor load of ATL in the murine model (Fig. 1B), and by the survival of the mice (Fig. 2A). When compared with the serum concentration of β 2 μ in the control group on days 14 and 25 after therapy, there was a significant reduction of the β 2 μ levels in the HAT, MAT, and 7G7/B6 treatment groups ($P < 0.0001$; Fig. 1B). Furthermore, there was a significant prolongation of survival of the mice in all of the antibody treatment groups when compared with that in the control group ($P < 0.01$; Fig. 2A). The mean survival duration of the control group was 27 days, whereas it was extended to 49 days in the HAT group, 47 days in the MAT group, and >64 days in the 7G7/B6 group (Fig. 2A). All of the mice in the control group had died by day 31 after therapy, whereas 14 of 15 of the HAT-treated and all of the MAT- and 7G7/B6-treated mice were alive at that time. In

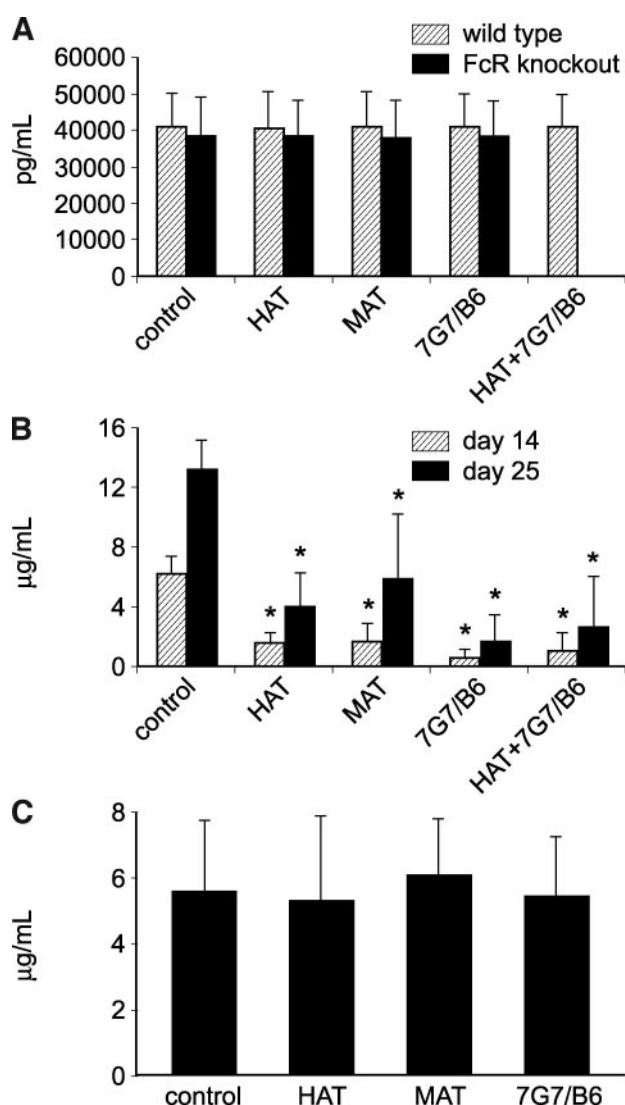


Fig. 1. Serum sIL-2R α and β 2 μ levels of the MET-1 leukemia-bearing SCID/NOD wild-type and SCID/NOD FcR $\gamma^{-/-}$ mice. The data represent the mean \pm SD. A, sIL-2R α levels in different groups at onset of the therapy. B, β 2 μ levels during the course of treatment in SCID/NOD wild-type mice. C, β 2 μ levels at day 14 after treatment in SCID/NOD FcR $\gamma^{-/-}$ mice. There was a significant reduction of β 2 μ levels in the HAT, MAT, and 7G7/B6 treatment groups in SCID/NOD wild-type mice as compared with that in the control group ($*P < 0.001$). However, the therapeutic efficacy was lost in SCID/NOD FcR $\gamma^{-/-}$ mice.

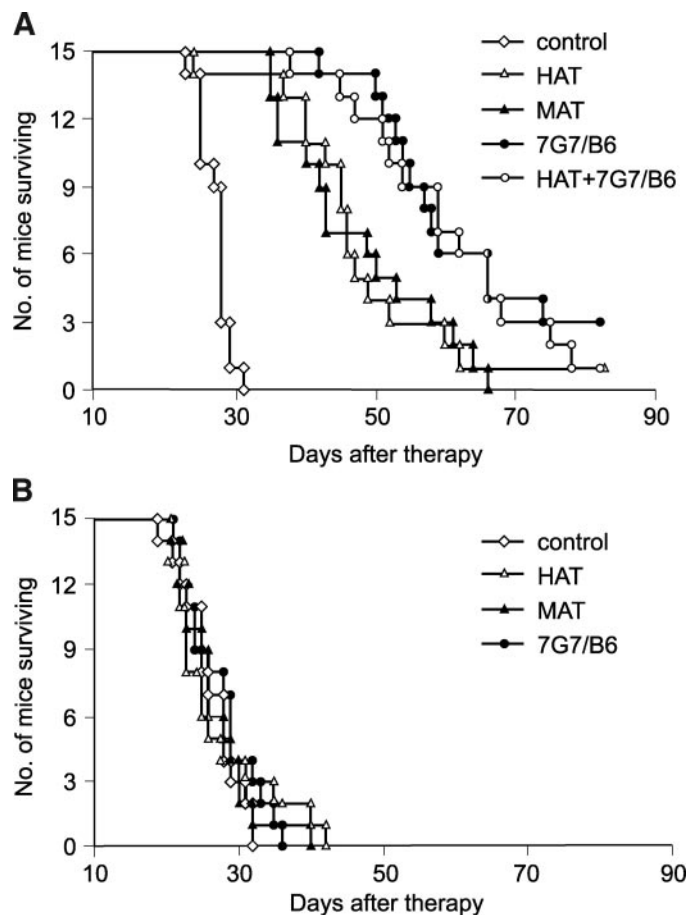


Fig. 2. Kaplan-Meier survival plot of the MET-1 leukemia-bearing SCID/NOD wild-type and FcR $\gamma^{-/-}$ mice. A, SCID/NOD wild-type mice. B, SCID/NOD FcR $\gamma^{-/-}$ mice. Treatment with HAT, MAT, 7G7/B6, and the combination of HAT and 7G7/B6 prolonged the survival of the MET-1 leukemia-bearing SCID/NOD wild-type mice significantly ($P < 0.01$). However, the therapeutic efficacy was lost in MET-1 leukemia-bearing SCID/NOD FcR $\gamma^{-/-}$ mice.

contrast, all of the antibodies used administered at the same dosing schedule as that in MET-1-bearing SCID/NOD wild-type mice did not show any therapeutic efficacy in SCID/NOD FcR $\gamma^{-/-}$ mice that were bearing the same quantity of MET-1 leukemic cells (Fig. 1A) as seen by the demonstration that there were no differences in the serum levels of the surrogate marker, $\beta 2\mu$ (Fig. 1C), and no difference in the survival of the leukemia-bearing mice (Fig. 2B) when compared with the control group.

Combination Therapy with HAT and 7G7/B6. HAT and 7G7/B6 recognize CD25 but define different epitopes on the molecule (14). The two antibodies bound to CD25-positive Kit225IG3 cells specifically, and they are not cross-competing (Fig. 3). The combination of these two antibodies for the management of ATL theoretically could manifest additive or synergistic efficacy. However, in this study, administration of 100 μ g of HAT and 100 μ g of 7G7/B6 weekly for 4 weeks did not show more benefit than that of 100 μ g of 7G7/B6 alone for the treatment of MET-1-bearing SCID/NOD wild-type mice as seen by the observation that there were no differences in the serum levels of $\beta 2\mu$ (Fig. 1B) and no difference in the survival of the mice between the 7G7/B6 and combination therapy groups (Fig. 2A).

Pharmacokinetics of Radiolabeled Antibodies. To determine whether there was any contribution provided by different concentrations of the antibodies in the blood to the different therapeutic efficacies observed with SCID/NOD wild-type and SCID/NOD FcR $\gamma^{-/-}$

mice, we performed a pharmacokinetics experiment with 125 I-labeled HAT, MAT, and 7G7/B6 in SCID/NOD wild-type and SCID/NOD FcR $\gamma^{-/-}$ mice. The blood clearance rates for each of the three antibodies evaluated in this study were similar in SCID/NOD wild-type and SCID/NOD FcR $\gamma^{-/-}$ mice (Fig. 4). The AUCs of the various 125 I-radiolabeled antibodies administered at a dose of 100 μ g were 2120 versus 1997%IDh/g for 125 I-HAT, 1598 versus 1651%IDh/g for 125 I-MAT, and 1085 versus 1227%IDh/g for 125 I-7G7/B6, respectively, for SCID/NOD wild-type versus SCID/NOD FcR $\gamma^{-/-}$ mice. There was no difference in AUC for each antibody between SCID/NOD wild-type and SCID/NOD FcR $\gamma^{-/-}$ mice (Fig. 4; $P > 0.1$). These results excluded the possibility of an exceptionally short survival of the monoclonal antibodies in SCID/NOD FcR $\gamma^{-/-}$ mice as the explanation for the failure to eliminate the tumor.

DISCUSSION

Thirty years after their discovery, monoclonal antibodies have become the most rapidly expanding class of pharmaceuticals for the management of a variety of human diseases (17–19). A major factor in the recent advances in the use of monoclonal antibodies directed toward cancer cells is the definition of meaningful antigenic targets. In the present study, we targeted CD25 (IL-2R α) with HAT, MAT, and 7G7/B6 monoclonal antibodies. The scientific basis for this strategy is that IL-2R α is not expressed by the majority of normal resting cells of the body but is expressed by malignant cells in select patients with ATL, cutaneous T-cell lymphoma, hairy cell B-cell leukemia, and Hodgkin's disease (5). In a clinical trial, 6 of 19 patients with ATL treated with the unmodified MAT developed a partial or complete remission lasting from 1 month to >11 years (13). In our laboratory, a preclinical *in vivo* murine model of ATL (MET-1) was developed to

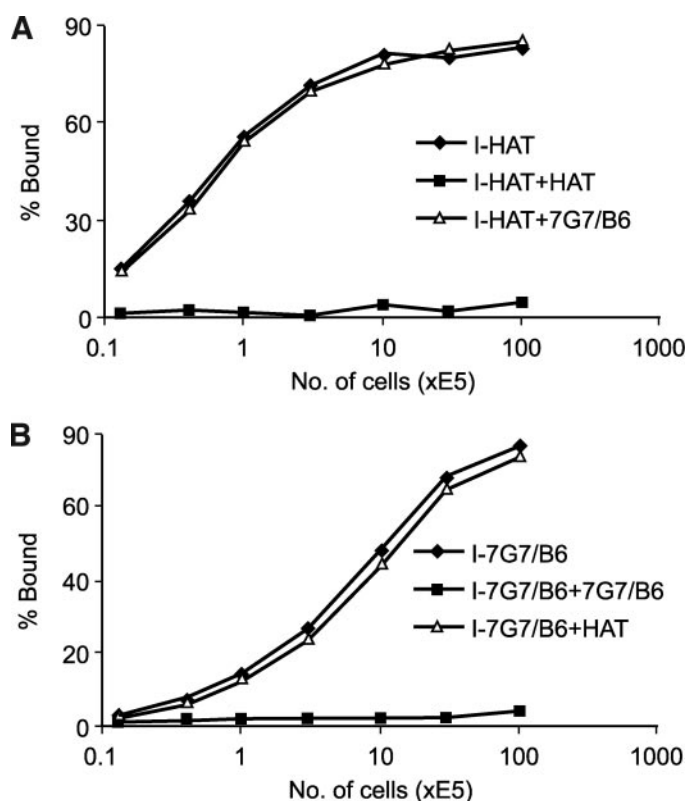


Fig. 3. Immunoreactivities of 125 I-HAT and 125 I-7G7/B6. A, 125 I-HAT with or without unlabeled HAT or 7G7/B6 inhibition. B, 125 I-7G7/B6 with or without unlabeled 7G7/B6 or HAT inhibition. 125 I-HAT and 125 I-7G7/B6 bound to CD25-positive Kit225IG3 cells specifically, and they did not cross-compete with each other.

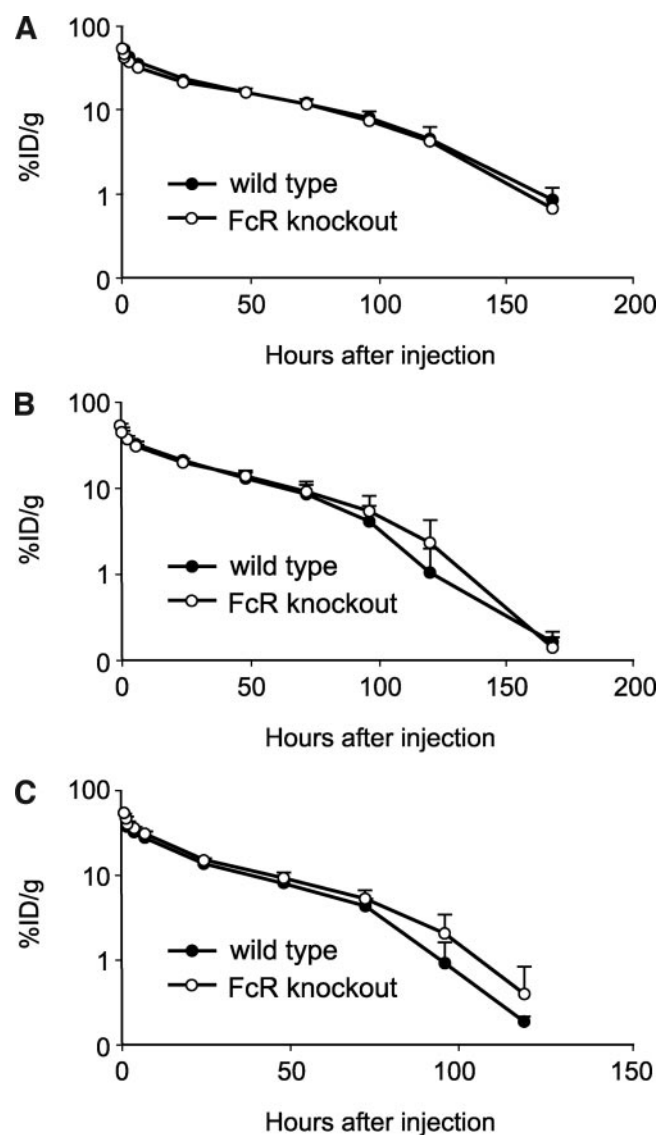


Fig. 4. Pharmacokinetics of ^{125}I -HAT, ^{125}I -MAT, and ^{125}I -7G7/B6 in SCID/NOD wild-type and SCID/NOD $\text{FcR}\gamma^{-/-}$ mice. The data represent the mean \pm SD. A, ^{125}I -HAT. B, ^{125}I -MAT. C, ^{125}I -7G7/B6. The blood clearance rates for each of the three radiolabeled monoclonal antibodies in SCID/NOD wild-type and SCID/NOD $\text{FcR}\gamma^{-/-}$ mice were similar. There was no difference in AUC for each antibody between SCID/NOD wild-type and SCID/NOD $\text{FcR}\gamma^{-/-}$ mice ($P > 0.1$).

test new therapeutic agents and to define their mode of action before initiating patient trials (6–11). In initial studies, antibodies to IL-2R α , including HAT, MAT, and 7G7/B6, inhibited the progression of leukemia and prolonged the survival of the mice (6). The present study was designed to define the mode of action of the anti-CD25 monoclonal antibodies in this model. Several mechanisms theoretically could be involved in the action of anti-IL-2R α in the MET-1 ATL model. These include blockade of the interaction of IL-2 with its growth factor receptor IL-2R α with the consequent cytokine deprivation-mediated cell death. This may be a major mode of action in patients with ATL because the majority of such patients responding to unmodified anti-CD25 antibodies manifest an autocrine IL-2/IL-2R α self-stimulatory loop. However, this mechanism does not provide an adequate explanation for the response of patients who are no longer in the IL-2-dependent phase of their leukemia and whose malignant cells no longer express IL-2 mRNA. Furthermore, cytokine deprivation does not represent an explanation for the efficacy in the MET-1 ATL model because the MET-1 leukemic cells do not express IL-2 mRNA

and do not respond to murine IL-2 (6). The therapeutically effective 7G7/B6 monoclonal antibody also does not bind to the IL-2 binding site of IL-2R α and thus does not block IL-2 interaction with its receptor (14). Although antibody-dependent cellular cytotoxicity mediated by NK cells may dominate in humans, such classical antibody-dependent cellular cytotoxicity mediated by NK cells also does not appear to be the mode of action of the monoclonal antibodies in this model because the SCID/NOD mice used in our study lack T and functional NK cells. Nevertheless, the antibodies appear to act through an FcR-dependent antibody-mediated mechanism. That is, although the three antibodies used showed significant inhibition of the MET-1 ATL growth in SCID/NOD wild-type mice, they showed no efficacy on the leukemia growth or animal survival in the ATL-bearing SCID/NOD $\text{FcR}\gamma^{-/-}$ mice. The lack of efficacy in the ATL-bearing SCID/NOD $\text{FcR}\gamma^{-/-}$ mice indicates that the expression of the receptor FcR γ III that requires the FcR γ chain probably is required for effective action of these antibodies directed toward CD25 in this *in vivo* model of ATL. This efficacy may represent a form of antibody-dependent cellular cytotoxicity requiring FcR γ receptors that are expressed on other cells, such as polymorphonuclear leukocytes and monocytes. Alternatively, the multimerization of the antibodies presented by the FcR on such cells may lead to aggregation of IL-2R α with consequent IL-2R α -mediated apoptotic cell death. In this regard, it should be noted that activation-induced cell death, a form of T-cell lymphocyte suicide, has as a required element the interaction of IL-2 with its private IL-2R α receptor, the target of the CD25-directed antibodies used in this study. IL-2R α -deficient mice do not manifest activation-induced cell death (20).

FcR γ III has been shown to be required for the action of an array of monoclonal antibodies in murine models of malignancy, including melanoma treated with antimelanoma monoclonal antibodies (21, 22), HER-2/*neu*-expressing breast tumors treated with trastuzumab (22), CD20-expressing B-cell lymphomas treated with rituximab (22), CD2-expressing T-cell malignancy, MET-1 treated with the anti-CD2 monoclonal antibody MEDI-507 (10), and CD52-expressing MET-1 leukemia treated with the anti-CD52 monoclonal antibody Campath-1 (11). However, the anti-CD30 monoclonal antibody Hefi-1 was effective in the karpas299 model of anaplastic large-cell lymphoma in SCID/NOD $\text{FcR}\gamma^{-/-}$ mice, suggesting in this case a direct apoptotic-inducing action of the anti-CD30 monoclonal antibody.⁵ The demonstration that Fc receptors presumably on granulocytes and monocytes is required for the action of the anti-CD25 antibodies examined in this study suggests that for their effective action there must be a proximity of FcR-expressing mononuclear cells to the leukemic cells, a requirement that may not be accomplished in patients with lymphomas, including ATL lymphoma.

An effort has been made to obtain synergistic improvement in the therapeutic efficacy in the MET-1 ATL murine model by combining two effective therapeutic agents (7–9). However, the simultaneous addition of two distinct monoclonal antibodies that require FcR γ expression yielded no additive effect. In particular, we saw no meaningful increased efficacy by the simultaneous administration of two distinct antibodies to CD25 in the present study or one to CD25 and a second to CD2 (10) or one to CD25 and a second to CD52 (11) in our previous trials. This suggests that the simultaneous administration of two antibodies that share a mode of action requiring FcR γ expression on effector cells does not increase the efficacy beyond that provided by saturating concentrations of a single monoclonal antibody. This observation contrasts with the synergistic efficacy observed when HAT was used in this model in concert with radioim-

⁵ Unpublished observations.

munotherapy or chemotherapeutic agents that have a distinct mode of action (7). This observation parallels those with trastuzumab and rituximab that provide additive or synergistic effects when combined with appropriate chemotherapeutic agents that have a markedly different mode of action from those of the monoclonal antibodies (23, 24).

In conclusion, FcR γ receptors on polymorphonuclear leukocytes and monocytes are required for the cytotoxic action of the three antibodies used that were directed toward CD25 in the MET-1 model of ATL. HAT is already in clinical trial for the treatment of patients with ATL or with other T-cell malignancies. The results of the present study and those previously performed with this model support the use of a combination regimen in a clinical trial involving patients with ATL using HAT in concert with effective chemotherapeutic agents or with other therapeutic approaches that have a different mode of action, such as radioimmunotherapy with monoclonal antibodies armed with radionuclides.

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