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Immunology

Complete suppression of *in vivo* growth of human leukemia cells by specific immunotoxins: Nude mouse models

(human T-cell leukemia/ascitic tumor/solid tumor/immunotherapy/ricin A chain-monoclonal antibody conjugates)

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ABSTRACT **In this study, immunotoxins containing monoclonal anti-human T-cell leukemia antibodies are shown to be capable of completely suppressing the tumor growth of human T-cell leukemia cells *in vivo* without any overt undesirable toxicity. These immunotoxins were prepared by conjugating ricin A chain (RA) with our monoclonal antibodies, SN1 and SN2, directed specifically to the human T-cell leukemia cell surface antigens TALLA and GP37, respectively. We have shown that these monoclonal antibodies are highly specific for human T-cell leukemia cells and do not react with various normal cells including normal T and B cells, thymocytes, and bone marrow cells. Ascitic and solid human T-cell leukemia cell tumors were generated in nude mice. The ascitic tumor was generated by transplanting Ichikawa cells (a human T-cell leukemia cell line) i.p. into nude mice, whereas the solid tumor was generated by transplanting s.c. MOLT-4 cells (a human T-cell leukemia cell line) and x-irradiated human fibrosarcoma cells into x-irradiated nude mice. To investigate the efficacy of specific immunotoxins in suppressing the *in vivo* growth of the ascitic tumor, we divided 40 nude mice that were injected with Ichikawa cells into four groups. Each group of 10 mice was injected with one of the following mixtures: (*i*) 40 μg of purified control mouse IgG [IgG1(κ)] (group 1), (*ii*) 40 μg of control RA conjugate (group 2), (*iii*) 20 μg of purified SN1 antibody [IgG1(κ)] and 20 μg of purified SN2 antibody [IgG1(κ)] (group 3), or (*iv*) 20 μg of SN1-RA and 20 μg of SN2-RA (group 4). Mice in groups 1 and 2 formed large ascitic tumors, and died 5.8–7.0 weeks after the transplantation. Group 3 mice also formed large ascitic tumors and died 6.4–7.8 weeks after the transplantation. However, none of the mice in group 4 that were treated with SN1-RA and SN2-RA showed any signs of a tumor or undesirable toxic effects for the 20 weeks that they were followed after the transplantation; these mice were indistinguishable from healthy control nude mice that were not injected with Ichikawa cells. Treatment with SN1-RA plus SN2-RA completely suppressed solid tumor growth in 4 of 10 nude mice carrying solid tumors and partially suppressed the tumor growth in the remaining 6 nude mice. These results strongly suggest that SN1-RA and SN2-RA may be useful for clinical treatment.**

Considerable expectation for the successful immunotherapy of cancer has been raised among many cancer researchers and clinicians since Kohler and Milstein (1) reported that somatic cell hybrids could be generated for the production of monoclonal antibodies (mAbs). At present, however, the results of cancer immunotherapy with mAbs have been, in general, rather disappointing. Reasons for the disappointing results have been reviewed in refs. 2 and 3. Some reasons relevant to the present study are as follows. (*i*) It is difficult to generate highly specific anti-human tumor mAbs. (*ii*) The *in vivo* cytotoxic effector mechanisms using antibodies (including mAbs) are not inherently efficient in killing the target tumor cells (2, 4, 5). (*iii*) Antigenic modulation of the target tumor cells may occur on the binding of mAbs, and the tumor cells may escape binding of subsequently administered mAbs (6, 7). This modulation may be, in some cases, a major mechanism by which tumor cells escape being killed by antibody. (*iv*) Therapeutic efficacy differs among mAbs of different isotypes and IgG subclasses (8–15).

To solve the first problem, we developed (16–20) an efficient system for generating highly specific anti-human tumor mAbs. The second, third, and fourth problems may be obviated by using an immunotoxin (IT) such as a mAb-ricin A-chain (RA) conjugate rather than unconjugated mAbs. In this IT, the mAb acts primarily as the specific delivery vehicle and RA acts as the cytotoxic effector. The enzymatically active RA is an extremely potent cytotoxic agent, when introduced into the cytoplasm of cells, that acts catalytically to inhibit protein synthesis (reviewed in refs. 21 and 22). Only one or a few molecules of RA are sufficient to kill the cell (23, 24). However, free RA *per se* is not significantly toxic to the cells because of its inability to efficiently bind to cell surfaces and to enter the cell. We have generated mAbs SN1 and SN2 that bind to two human T-cell leukemia-associated cell surface glycoproteins TALLA (17, 25) and GP37 (18, 19), respectively. When these mAbs were conjugated to RA, the IT generated was highly specific in killing T-cell leukemia cells *in vitro* (26, 27). In the present study we report the *in vivo* cytotoxic activity of these ITs.

MATERIALS AND METHODS

Mice. Six- to 8-week-old female BALB/c (*nu/nu*) mice were obtained from Harlan Sprague Dawley (Indianapolis, IN) through the Frederick Cancer Research Facility of the National Cancer Institute. The mice were housed under sterile conditions in cages with filter bonnets in a laminar flow unit (Lab Products, Maywood, NJ). They were given sterilized pellet food and tap water.

Cell Lines. Ichikawa, a human T-cell leukemia cell line, was kindly provided by K. Minato and M. Shimoyama of the Japanese National Cancer Center Hospital (Tokyo); and HT-1080, a human fibrosarcoma cell line, was kindly provided by S. Rasheed of the University of Southern California. Human T-cell leukemia cell lines, MOLT-4, JM, and Ichikawa, were cultured in RPMI 1640 medium supplemented with 4% (vol/vol) heat-inactivated fetal calf serum as described (28). HT-1080 cells were grown in monolayer culture in Dulbecco's modified Eagle's medium (GIBCO) supplemented with 10% (vol/vol) fetal calf serum and gentamicin (50 μg/ml).

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Abbreviations: mAb, monoclonal antibody; IT, immunotoxin; RA, ricin A chain.

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Reagents. *N*-Succinimidyl-3-(2-pyridyldithio)propionate was purchased from Pharmacia. Purified RA was obtained from E-Y Laboratories (San Mateo, CA). The ^{3}H-labeled L-amino acid mixture (15 different [^{3}H]amino acids) were purchased from ICN.

Purified IgGs of mAbs and Control Mouse IgG. The IgG was isolated from BALB/c murine ascites containing mAbs SN1 [IgG1(κ)] or SN2 [IgG1(κ)] by DE52 chromatography and gel filtration on Sephadex G-150 (26). Control mouse IgG was isolated similarly from BALB/c ascites containing MOPC-195 variant plasmacytoma IgG [IgG1(κ)].

Preparation of ITs and Control Conjugate. The purified IgGs of mAbs or control murine ascites were conjugated with RA using *N*-succinimidyl-3-(2-pyridyldithio)propionate as described (26). Briefly, purified IgGs in PBS (0.01 M sodium phosphate, pH 7.4, containing 0.15 M NaCl) were treated with a 15-fold molar excess of *N*-succinimidyl-3-(2-pyridyldithio)propionate for 30 min at room temperature to introduce 2-pyridyl disulfide groups into the IgG molecule. The modified and dialyzed proteins were then mixed with a 3-fold molar excess of the purified, freshly reduced RA in PBS containing 1 mM EDTA and incubated at 4°C for 15 hr. The IgG-RA conjugates were separated from the unbound RA by gel filtration on a calibrated Sephacryl S-200 column. The conjugates were stored at −76°C after penicillin (100 units/ml), streptomycin (100 μg/ml), and gentamicin (50 μg/ml) were added.

Inhibition of Protein Synthesis by ITs. This test was carried out as described (26). In some tests, 10 mM NH_4Cl was included to potentiate the action of the ITs.

Transplantation of MOLT-4 and Ichikawa Cells into Nude Mice. MOLT-4 cells were transplanted by the procedures of Ziegler *et al.* (29) and Dillman *et al.* (30). Briefly, HT-1080 cells were harvested after treatment with 0.2% trypsin, washed extensively, and x-irradiated with 6000 rad (1 rad = 0.01 Gy). MOLT-4 cells were mixed with a 4-fold excess of x-irradiated HT-1080 cells, and the mixture was washed and resuspended in saline to obtain a final concentration of 10^8 mixed cells per ml. The cell mixture (0.1 ml) was injected s.c. into the right flank of a nude mouse that had been x-irradiated weekly with 200 rad for 3 weeks; the injection was carried out 3 days after the final irradiation. Under these conditions, 23 of 28 mice developed solid tumors. We followed the local tumor growth by measuring two perpendicular diameters. The Ichikawa cell suspension (1 ml with 1.6×10^7 cells) in saline was injected i.p. into a nude mouse to form an ascitic tumor (31). All 35 of the 35 mice injected developed ascitic tumors.

Treatment of Solid Tumor with ITs. Palpable tumors of 0.3–0.7 cm^2 size that developed 2–3 weeks after inoculation of MOLT-4 and HT-1080 cells were treated by intratumoral injections of SN1-RA plus SN2-RA in PBS or of a control MOPC-195 Var-RA in PBS. This treatment was repeated on days 4, 7, and 10. Tumor size was determined by measuring two perpendicular diameters with calipers and expressed as $S = \pi ab$, where S is the tumor surface area (cm^2), a is the major axis (cm), and b is the minor axis (cm).

Treatment of Ascitic Tumor with ITs. In the initial experiment, a nude mouse was injected i.p. with PBS containing 20 μg of SN1-RA and 20 μg of SN2-RA or with 40 μg of control conjugates 2 hr after the injection of Ichikawa cells. The injection of ITs was repeated on day 4. In the subsequent experiment, four groups of nude mice were injected i.p. (*i*) with 40 μg of purified control mouse IgG (MOPC-195 Var), (*ii*) with 40 μg of control RA conjugate (MOPC-195 Var-RA), (*iii*) with 20 μg of purified SN1 and 20 μg of SN2, or (*iv*) with 20 μg of SN1-RA and 20 μg of SN2-RA. The IT injection was repeated twice on days 4 and 28.

Cellular RIA. The presence (or absence) of leukemia cells in the peritoneal fluid of IT-treated mice and control healthy mice was investigated by a cellular RIA that was based on modifications of our assay (17, 18). Briefly, 10 μl containing 5×10^5 cells in Hepes buffer and 0.1% human IgG (in triplicate) and 20 μl of ^{125}I-labeled SN1 or ^{125}I-labeled SN2 in Hepes buffer were incubated in individual wells of 96-well microtiter plates for 60 min at 4°C. The Hepes buffer contained RPMI 1640 medium/25 mM Hepes, pH 7.3/0.5% bovine serum albumin/Trasylol at 50 kallikrein units per ml/0.1% NaN_3. The mixtures were centrifuged at 500 × *g* and 4°C for 10 min, and the pelleted cells were washed three times. The radioactivity in the washed pellet was determined in a γ-ray spectrometer. As additional controls, cultured Ichikawa cells, MOLT-4 cells, and CCRF SB cells (a negative control) were included in the assay.

Histology. Sections from the tumors, mesenterium, lymph nodes, spleens, kidneys, livers, lungs, and brains were kept frozen until used. Sections were cut on a cryostat, stained with hematoxylin–eosin, and examined by light microscopy.

RESULTS

***In Vitro* Cytotoxic Activity of ITs.** The *in vitro* ability of SN1-RA and SN2-RA to inhibit protein synthesis in T-cell leukemia cells was determined as described (26). The results are shown in Fig. 1. SN1-RA and SN2-RA could nearly completely inhibit protein synthesis in JM cells at IT concentrations of $10^{-7.5}$ M and 10^{-8} M, respectively. These results are consistent with our results that were obtained using different preparations of SN1-RA and SN2-RA (26). The inhibitory activity of SN1-RA and SN2-RA was significantly enhanced by 10 mM NH_4Cl (Fig. 1), which agrees with our results obtained using colony-forming unit assays (27) and which is also consistent with reports by others (32–36).

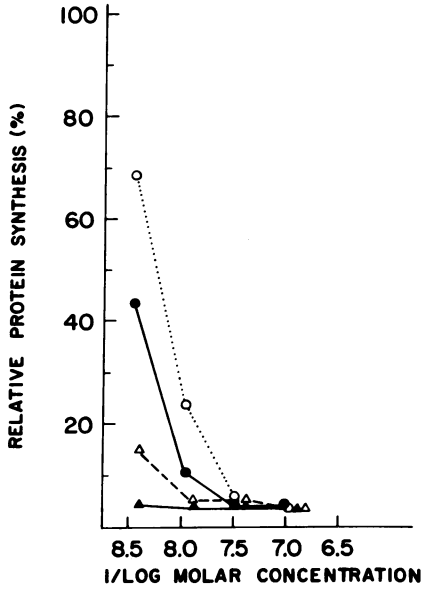

FIG. 1. Inhibition of protein synthesis in JM cells by ITs SN1-RA (○, ●) and SN2-RA (△, ▲). The leukemia cells were incubated with various concentrations of the conjugates for 21 hr in the absence (○, △) or presence (●, ▲) of 10 mM NH_4Cl, a potentiator of ITs. The incubated cells were centrifuged, washed, and resuspended in amino acid-free (except for glutamine) medium containing 1.5 μCi (1 Ci = 37 GBq) of a ^{3}H-labeled amino acid mixture. The cell suspension was incubated for 2 hr and centrifuged. The resulting cell pellet was washed extensively. Then, the radioactivity in the trichloroacetic acid-insoluble material in the washed cell pellets was determined. Protein synthesis in the conjugate-treated cells is expressed as the percentage of [^{3}H]amino acids incorporated into the control cells not exposed to conjugate.

Transplantation of Human T-Cell Leukemia Cells into Nude Mice. MOLT-4 and Ichikawa cells were successfully transplanted into nude mice and formed solid and ascitic tumors, respectively. Twenty-three of 28 mice inoculated s.c. with MOLT-4 cells developed solid tumors, whereas all 35 mice inoculated i.p. with Ichikawa cells developed ascitic tumors. It should be noted that both MOLT-4 and Ichikawa cells express two T-cell leukemia-associated cell surface glycoproteins TALLA and GP37 that were defined by mAbs SN1 and SN2, respectively (refs. 17 and 18; unpublished results).

Complete *In Vivo* Suppression of Ascitic Tumor Growth. In a preliminary test, five nude mice that received Ichikawa cells were injected twice i.p. with 20 μg of SN1-RA and 20 μg of SN2-RA. As a control, another five recipient nude mice were injected twice with 40 μg of the control conjugate MOPC-195 Var-RA. All the control mice died 6–7 weeks after Ichikawa cell transplantation. In contrast, no tumor growth was observed in the five nude mice treated with SN1-RA and SN2-RA during the same period. One of the five mice, however, developed a tumor 7 weeks later and died. Based on this initial result, we carried out the following experiment.

We divided 40 nude mice that received Ichikawa cells into four groups. Each group of 10 mice was injected three times i.p. (*i*) with 40 μg of purified control mouse IgG (MOPC-195 Var) (group 1), (*ii*) with 40 μg of control RA conjugate (MOPC-195 Var-RA) (group 2), (*iii*) with 20 μg of purified SN1 antibody and 20 μg of purified SN2 antibody (group 3), or (*iv*) with 20 μg of SN1-RA and 20 μg of SN2-RA (group 4). Mice of groups 1 and 2 formed large ascitic tumors (Fig. 2*A*), and all died 5.8–7.0 weeks after the transplantation of the Ichikawa cells. The median survival time of group 1 mice treated with control IgG was 44.5 days and that of group 2 mice treated with control RA conjugate was about the same (Fig. 3). Treatment of tumor-bearing mice with SN1 and SN2 mAbs (group 3) extended their median survival time only slightly, i.e., 5.5 days, and all 10 mice died 6.4–7.8 weeks after transplantation of the Ichikawa cells. In contrast, none of the group 4 mice that were transplanted with Ichikawa cells and treated with the specific anti-T-cell leukemia ITs SN1-RA and SN2-RA developed any tumor, and they were indistinguishable from healthy normal nude mice (Fig. 2*B*). We did not detect any sign of tumor or of undesirable overt toxicity in the 10 mice for the 20 weeks that they were followed (see Fig. 3 and below). To confirm the absence of tumor in the group 4 mice, the 10 mice were sacrificed and the peritoneal fluid cells were tested by a cellular RIA using

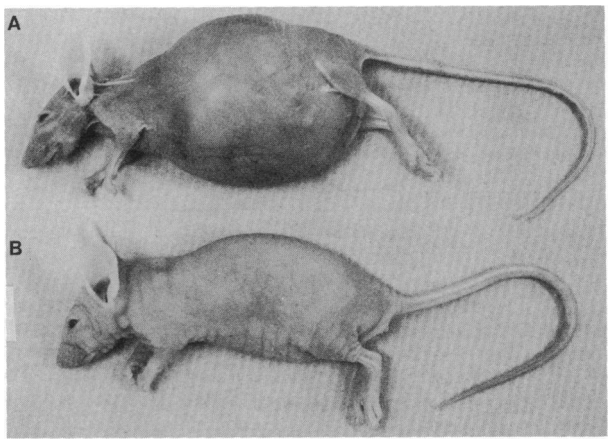

FIG. 2. Specific effect of SN1-RA and SN2-RA on suppressing ascitic tumor of Ichikawa cells in nude mice. Ichikawa cell-bearing mice were treated with control RA conjugate (40 μg) (MOPC-195 Var-RA) (*A*) or SN1-RA (20 μg) plus SN2-RA (20 μg) (*B*). The picture was taken 6 weeks after the inoculation with Ichikawa cells. The mouse shown in *B* was indistinguishable from normal healthy mice.

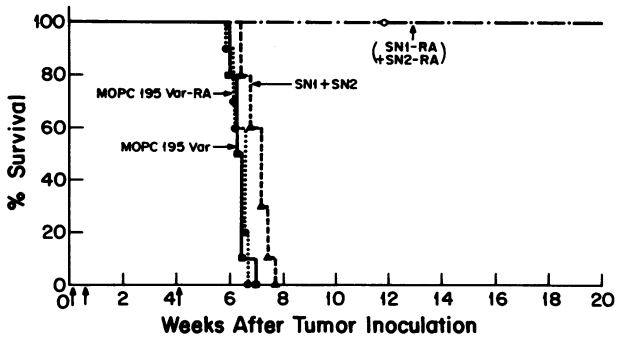

FIG. 3. Complete *in vivo* suppression of ascitic tumor growth by specific ITs. Four groups (10 mice per group) of nude mice inoculated i.p. with Ichikawa cells were treated with (*i*) mouse control IgG (40 μg) (MOPC-195 Var), (*ii*) control RA conjugate (40 μg) (MOPC-195 Var-RA), (*iii*) SN1 (20 μg) plus SN2 (20 μg) antibodies, or (*iv*) SN1-RA (20 μg) plus SN2-RA (20 μg). The injection of the IgGs and RA conjugates was done 2 hr, 4 days, and 28 days after the transplantation of Ichikawa cells.

^{125}I-labeled SN1 and ^{125}I-labeled SN2. No tumor cells were detected in any of the samples. The absence of tumor or undesirable toxicity in the SN1-RA- and SN2-RA-treated nude mice was further corroborated by histological examination of the various solid tissues (data not shown). The tissues examined were mesenterium, lymph nodes, spleens, kidneys, livers, lungs, and brains.

In conclusion, our specific anti-human leukemia ITs SN1-RA plus SN2-RA completely suppressed tumor growth in 10 of 10 nude mice without showing any undesirable overt toxicity. It is remarkable that little intragroup variation was observed for all four groups of nude mice investigated (Fig. 3).

Suppression of Growth of Solid Tumor. Solid tumors were generated by s.c. inoculating x-irradiated nude mice with MOLT-4 cells and with x-irradiated human fibrosarcoma cells (HT-1080 cells). Tumors became distinguishable (0.3–0.7 cm^2 in surface area) 2–3 weeks after the inoculation of MOLT-4 and HT-1080 cells. The tumor continued to grow resulting in death; the tumor was well encapsulated and located at the inoculation site. Twenty nude mice bearing solid tumors of 0.3–0.7 cm^2 in surface area were divided into four groups. Each group of five mice was treated (*i*) with 2 μg of control MOPC-195 Var-RA conjugate, (*ii*) with 20 μg of MOPC-195 Var-RA, (*iii*) with 1 μg of SN1-RA and 1 μg of SN2-RA, or (*iv*) with 10 μg of SN1-RA and 10 μg of SN2-RA. The tumors in mice of groups 1 and 2 grew rapidly (Figs. 4 and 5 *a* and *b*), and all 10 mice died 5–8 weeks after the initial treatment with control RA conjugate. Among the mice of group 3 that were treated with 1 μg of SN1-RA and 1 μg of SN2-RA, two became free of tumor (Fig. 5*c*), whereas the tumor growth in the remaining three mice was suppressed

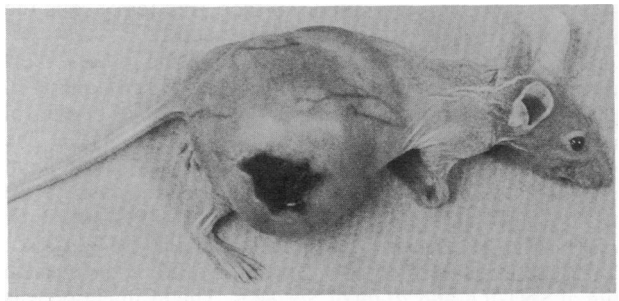

FIG. 4. Nude mouse bearing a solid tumor of MOLT-4 cells. The mouse bearing the solid tumor was injected with 2 μg of control RA conjugate. The picture was taken 68 days after the transplantation.

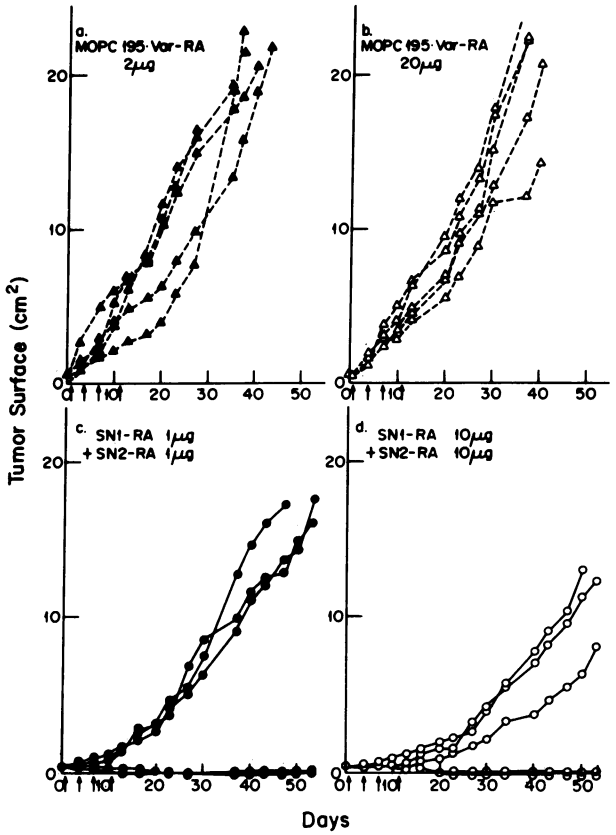

FIG. 5. Complete and partial suppression of solid tumor growth of MOLT-4 cells in nude mice by specific ITs. Twenty nude mice bearing distinct solid tumors were divided into four groups, and each group of mice was injected intratumorally with 2 (*a*) or 20 μg (*b*) of control RA conjugate or with 1 (*c*) or 10 μg (*d*) of SN1-RA and 1 (*c*) or 10 μg (*d*) of SN2-RA. The injection of control RA conjugate and specific ITs was repeated 4, 7, and 10 days after the initial injection. The initial injection was carried out 2–3 weeks after the transplantation of MOLT-4 into the nude mice, when the solid tumor became distinct. The abscissa represents days after the initial injection of ITs and control conjugate. The ordinate represents the tumor surface area.

only during the time the four injections of SN1-RA plus SN2-RA were given; the tumor began to grow rapidly soon after the final injection (Fig. 5*c*). Among the group 4 mice that were treated with 10 μg of SN1-RA and 10 μg of SN2-RA, complete suppression of tumor growth was observed for two mice (Figs. 5*d* and 6) and partial suppression was observed for the remaining 3 mice (Fig. 5*d*). We examined the histology of the liver, spleen, and kidney of the two mice whose tumors regressed completely; these tissues were indistinguishable from the corresponding tissues of healthy control nude mice that were not injected with any IT.

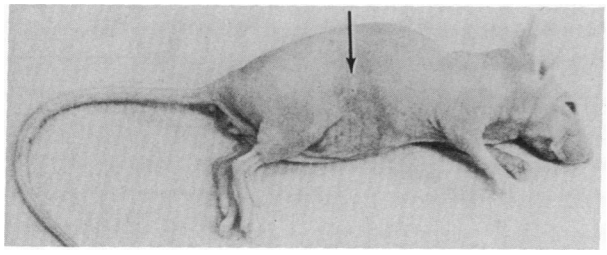

FIG. 6. Complete *in vivo* suppression of solid tumor growth of human T-cell leukemia cells by specific ITs. A nude mouse bearing a distinct MOLT-4 cell solid tumor was injected four times intratumorally with 10 μg of SN1-RA and 10 μg of SN2-RA. The tumor regression was observed between 17 and 20 days after the initial injection, and the mouse became free of tumor. The picture was taken 58 days after the initial injection of ITs. Only a scar remains at the site of the original tumor.

DISCUSSION

There are many publications that report *in vitro* cytotoxic activity of ITs (reviewed in ref. 22). However, there are relatively few reports concerning *in vivo* cytotoxic activity of ITs. Krolick *et al.* (37) and Seto *et al.* (38) reported that their ITs containing RA were effective in *in vivo* suppression of mouse tumors grown in syngeneic mice. Partial suppression of *in vivo* growth of guinea pig and mouse tumors was reported by other investigators using ITs containing diphtheria toxin A chain, pokeweed antiviral protein, and saporin (39–41). Weil-Hillman *et al.* (42) used ITs containing intact ricin and RA to treat a solid CEM tumor (a human T-cell leukemia cell line) in nude mice. In the case of the intact ricin-containing IT, nonselective binding of the IT through the ricin B chain to the irrelevant cells was blocked by systemic i.v. administration of lactose and by local intratumoral injection of lactose with the intact ricin-containing IT. Although the intact ricin-containing IT was effective in suppressing the CEM tumor, it still had substantial nonspecific cytotoxicity. On the other hand, their RA-containing IT was not effective in suppressing the CEM tumor. Laurent *et al.* (43) injected a RA-containing IT into two leukemia patients; one with acute T-lymphoblastic leukemia and the other with chronic B-lymphocytic leukemia. Their IT was not effective in either patient. It should be noted that both Weil-Hillman *et al.* (42) and Laurent *et al.* (43) used mAb T101 in preparing their ITs. T101 is directed to an abundant normal T-cell and thymocyte antigen that is also present on malignant T cells and chronic B-lymphocytic leukemia cells (44, 45). Several investigators studied T101-containing ITs, and such studies provided information important in understanding various parameters for the cytotoxic action of ITs (35, 42, 43, 46–49). However, the *in vivo* clinical utility of T101-containing IT will be limited because of its reactivity with normal T cells and thymocytes. This reactivity will not only result in killing the normal cells but also make it extremely difficult to eradicate residual leukemia cells in the presence of the T101-containing IT-reactive excess normal cells. In contrast, our mAbs, SN1 and SN2, are highly specific for T-cell leukemia cells and do not react with various normal cells including normal T and B cells, thymocytes, and bone marrow cells (17–19, 25). SN1 did not react with any normal cells and tissues tested (17, 25), whereas SN2 showed a very weak reactivity with normal platelets as detected by a sensitive cellular RIA (18, 19); this reaction was not detectable by fluorescence-activated cell sorter analysis using a sensitive phycoerythrin-labeled antibody (19). Furthermore, ITs prepared by conjugating purified mAbs SN1 and SN2 with RA has been shown to be highly specific in killing T-cell leukemia cells *in vitro* (26, 27).

In the present work, the *in vivo* cytotoxic activity of SN1-RA and SN2-RA was studied using two model systems, nude mice with ascitic or solid tumors. The ascitic tumor obviously is closer to human leukemia than is the solid tumor. Nevertheless, the test results obtained using the solid tumor may provide valuable information for treating various human solid tumors with ITs also. The results obtained using the ascitic tumor system are remarkably good in that SN1-RA plus SN2-RA completely suppressed the tumor growth of human T-cell leukemia cells without showing any overt toxicity in all nude mice tested, whereas control RA conjugate was not effective at all in suppressing the tumor (Figs. 2 and 3). The purified mAbs SN1 and SN2 were only

marginally effective (Fig. 3). These results indicate a strong potential of SN1-RA and SN2-RA for treating T-cell leukemia patients. With regard to the solid tumor, complete suppression was observed for 4 of 10 nude mice and partial suppression was observed for the remaining 6 nude mice. The data presented in this report provide strong evidence that ITs indeed have good potential for *in vivo* therapy of human diseases if proper antibodies are used in preparing the ITs.

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