Treatment of B-Cell Lymphoma With Chimeric IgG and Single-Chain Fv Antibody–Interleukin-2 Fusion Proteins

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Anti-idiotype (Id) antibodies (Abs) have been shown to be effective in treatment of B-cell lymphoma in animal models and in clinical trials. The combination of interleukin-2 (IL-2) can augment the therapeutic effect of anti-Id Abs. To further improve the power of the combined therapy, a monoclonal anti-Id Ab, S5A8, specifically recognizing a murine B-cell lymphoma 38C13, was genetically modified to contain the IL-2 domain and thus use the unique targeting ability of Abs to direct IL-2 to the tumor site. Two forms of the anti-Id–IL-2 fusion proteins were constructed: one configuration consisting of mouse-human chimeric IgG (chS5A8–IL-2) and the other containing only the variable light (VL) and variable heavy (VH) Ab domains covalently connected by a peptide linker (scFvS5A8–IL-2). Both forms of the anti-Id–IL-2 fusion proteins retained IL-2 biological activities and were equivalent in potentiating tumor cell lysis in vitro. In contrast, the antigen-binding ability of scFvS5A8–IL-2 was 50- to 40-fold lower than that of the bivalent chS5A8–IL-2. Pharmacokinetic analysis showed that scFvS5A8–IL-2 was eliminated about 20 times faster than chS5A8–IL-2. Finally, it was shown that chS5A8–IL-2 was very proficient in inhibiting 38C13 tumor growth in vivo, more effectively than a combined therapy with anti-Id Abs and IL-2, whereas scFvS5A8–IL-2 did not show any therapeutic effect. These results demonstrate that the anti-Id–IL-2 fusion protein represents a potent reagent for treatment of B-cell lymphoma and that the intact IgG fusion protein is far more effective than its single-chain counterpart.

THE ABILITY OF monoclonal antibody (MoAb) to specifically localize in tumor tissues in vivo offers an attractive therapeutic approach for cancer therapy. However, natural effector functions of Abs such as complement activation or Ab-dependent cellular cytotoxicity (ADCC), although effective in mediating tumor cell destruction in vitro, are generally not sufficient to completely suppress tumor cell growth in vivo.1 Treatment with MoAb alone has only achieved very limited success in the clinic.1 Interleukin-2 (IL-2) is a potent biological mediator of the immune system and has demonstrated the ability to augment ADCC2 and potentiate the antitumor activity of natural killer (NK) cells3-5 in vitro. Preclinical studies showed that application of IL-2, either independently or combined with infusion of lymphokine-activated killer cells, resulted in the induction of a long-lasting antitumor response, leading to the rejection of an otherwise lethal tumor.6-8 However, clinical experiences of IL-2 therapy were not as encouraging as the preclinical studies, only producing responses in some patients with melanoma and renal cell carcinoma.9,10 The poor clinical responses were in part due to failure to achieve long-lasting therapeutic concentrations in the tumor and by the severe systemic toxicity associated with high-dose IL-2 therapy.11,12

Under physiological conditions, cytokines usually exert their effector functions in a paracrine fashion. It is generally thought that specifically targeting IL-2 to the tumor microenvironment not only would achieve better antitumor activity, but also would help alleviate IL-2 toxicity. To achieve a local concentration of IL-2 at the tumor site, IL-2 has been applied either intratumorally13 or in a form of a minipellet that slowly releases IL-2 by diffusion and dissolution.14 Both methods resulted in consistent but limited inhibition of tumor growth. Another approach to generate high concentrations of specific cytokines at the tumor site involved the transduction of tumor cells with cytokine genes.15,16 The sustained release of cytokines by such cytokine-secreting tumor cells produced dramatic local inflammatory response without significant systemic toxicity and often resulted in the ultimate destruction of the transduced tumor cells. In some cases, systemic immunity was induced against wild-type parent tumors challenged at distant locations.17,18 Fusion proteins consisting of Ab and cytokine molecules have been
were specifically directed to the tumor site. To this end, we made two forms of anti-Id-IL-2 fusion proteins; one configuration consisting of mouse-human chimeric IgG and the other containing a smaller single-chain (scFv) Ab comprising linked variable light (V_L) and variable heavy (V_H) Ab domains. The in vitro characterization and in vivo therapeutic efficacy of these two forms of anti-Id-IL-2 fusion proteins were compared.

MATERIALS AND METHODS

Mice. Female C3H/HeN mice, 6 to 8 weeks old, were purchased from National Laboratory Animal Breeding and Research Center (Taipei, Taiwan) and housed at the Laboratory Animal Facility, Institute of Biomedical Sciences, Academia Sinica (Taipei, Taiwan).

Cell lines. 38C13 murine B-cell lymphoma is a carcinoma (DBMA)-induced tumor originally produced in a T-cell–depleted C3H/eB mouse.27 V1-1 is a subclone of an Id-negative variant V1 that was derived from the original 38C13 tumor by selection with passive Ab therapy in vivo.28 A1-2 is a hybridoma cell line that secretes 38C13 Id (IgM, κ).29 SSaA is a hybridoma-secreting MoAb (IgG2b) specifically against 38C13 Id.29 HT-2 is a murine helper T-cell line dependent on IL-2 or IL-4 for growth. Cells were maintained in RPMI 1640, 10% heat-inactivated fetal calf serum (FCS), 2 mmol/L L-glutamine, 100 U/mL penicillin, 100 μg/mL streptomycin, and 50 μmol/L 2-ME (RPMI-10) at 37°C, 5% CO2 in a humidified incubator. HT-2 cells were grown in the above-noted medium supplemented with 1% supernatant of a transfected cell line producing Id-IL-2 fusion protein.30 SF21 insect cells were cultured at 27°C in TMN-FH medium (PharMingen, San Diego, CA) containing 10% heat-inactivated FCS.

Construction and production of chimeric IgG anti-Id-IL-2 fusion protein. The V_H and V_L genes of SSaA were obtained by reverse transcription and polymerase chain reaction (PCR) amplification using upstream primers containing an NcoI site overlapping the translation start codon ATG and downstream primers containing KpnI sites located in the 3′ segments. The NcoI-KpnI fragments were gel-purified and replaced the corresponding V_H and V_L genes in the heavy-chain (p3079) and light-chain (p3077) expression vectors as previously described.31 The resulting light-chain plasmid, p301, contained the V_HSSaA joined to the human κ constant region gene (C_k), and the heavy-chain plasmid, p302, contained the V_HSSaA joined to the human γ1 constant region gene (C_γ1). To construct the anti-Id-IL-2 fusion protein, V_HSSaA was used to replace the V_H gene in plasmid p316330 to produce plasmid p119 containing the human C_γ1 gene followed by two gly codons and the mature murine IL-2 sequence. The heavy-chain vector (p302 or p119) was then cotransfected with the light-chain vector, p3112, containing the V_L gene from a human B-cell lymphoma (RF) joined to the human C_k gene.32 Transfected clones were expanded for large-scale production in RPMI 1640 containing 1% low IgG FCS and purified by protein A chromatography. Bound Abs were eluted from the column with buffer containing 0.1 mol/L glycine, 0.15 mol/L NaCl, pH 2.4, and brought to neutral pH with 0.5 mol/L sodium phosphate, pH 8. Purified proteins were dialyzed extensively versus phosphate-buffered saline (PBS) and sterilized by filtration. The concentrations of purified Abs were determined by a bicinchoninic acid-based protein assay (Pierce, Rockford, IL).

Construction and production of scFv anti-Id-IL-2 fusion protein. A SSaA scFv in V_L–V_H orientation was assembled by overlapping PCR, as previously described,33 with the 14 amino acid 212 linker.34 GSTSSGKKSSEGKKG, covalently bridging the V_L and V_H domains. The SSaA scFv PCR fragment was then digested with BamHI and EcoRI and gel-purified before ligation into the polycloning region of the baculovirus expression vector pAcGFP67B (PharMingen), which contains the signal peptide of the glycoprotein gp67 of the baculovirus strain AcNPV to facilitate protein secretion. The fragment containing a mature mouse IL-2 sequence was generated by PCR from plasmid pmut-1 (ATCC 37553; American Type Culture Collection, Rockville, MD). The upstream primer contained two Gly codons 3′ to the EcoRI site, and the downstream primer contained a stop codon 5′ to the PstI site. The PCR product was then digested with EcoRI and PstI, gel-purified, and ligated to the 3′ end of the scFv in the expression vector to produce plasmid p221. To develop the recombinant baculovirus, SF21 cells were cotransfected with plasmid p221 and linearized BaculoGold viral DNA (PharMingen) according to the manufacturer’s instructions. Five days after transfection, supernatants containing the recombinant viruses were harvested and used to infect a monolayer of SF21 cells seeded at 9 × 10^6 per well in a 6-well tissue culture plate. After 1 hour of incubation at 27°C, supernatants were removed and cells were overlaid with 1.5 mL of 0.8% L.M.P agarose (GIBCO BRL, Gaithersburg, MD). The putative recombinant viral plaques were isolated and tested for Ab production. Clones producing the highest levels of proteins were selected, expanded to produce large amounts of scFv anti-Id–IL-2 fusion protein, and purified by affinity chromatography prepared with 38C13 Id purified as previously described.35

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblot analysis. SDS-PAGE and transfer of proteins to nitrocellulose by semidry electroblotting were performed as previously described.36 Blots were probed with either 38C13 Id at 1 μg/mL or rat antinouse IL-2 MoAb (JES6-1A12; IgG2a; PharMingen) at 1:500. The bound 38C13 Id and anti–IL-2 Ab were then reacted with horseradish peroxidase-conjugated goat antirat IgG (1:1,000; Cappel, Organon Teknika, Veeldijk, Belgium) and goat antirat IgG (1:1,000; Cappel, respectively). Blots were developed by the enhanced chemiluminiscence Western blot detection system (Amersham, Little Chalfont, UK).

Antigen binding assay. The Id-binding ability of the various anti-Id Abs were determined by a competitive enzyme-linked immunosorbent assay (ELISA) binding assay.37 Wells of the microtiter plates were coated with 1 μg/mL of purified 38C13 Id and blocked with 10% bovine calf serum in PBS (BCS/PBS). The unlabeled anti-Id Abs at a concentration of 50 nmol/L were applied to the first row of microtiter plates and then titrated by a factor of 1:3 with 10% BCS/PBS so that 5 logs in concentration could be analyzed. The final volume was 100 μL in each well. One hundred microliters of biotinylated SSaA was immediately added to each well to reach a final concentration of 0.1 nmol/L and incubated overnight at 4°C. The bound biotinylated SSaA was then detected with alkaline phosphatase-conjugated streptavidin (PharMingen), developed with p-nitrophenyl phosphate (Sigma, St Louis, MO) as the substrate, and absorbance at 405 nm was measured using an ELISA plate reader. The inhibition of biotinylated SSaA binding by each anti-Id Abs was calculated relative to the biotin binding in the absence of competing Ab. The inhibition curves were plotted and the 50% inhibition value was calculated for each tested Ab.

Fluorescence-activated cell sorting (FACS) analysis. 38C13 or V1-1 cells (1 × 10^5) were reacted at 4°C with 10 μg/mL of 55A8, chSSaA, chSSaA–IL-2, or scFvSSaA–IL-2. An irrelevant mouse IgG2b MoAb was included as a control. The bound SSaA and the control mouse Ab were detected with fluorescein isothiocyanate (FITC)-labeled goat antirat Ab (1:100; Sigma). The bound chSSaA was detected with FITC-labeled goat antihuman κ Ab (1:100; Cappel). The bound chSSaA–IL-2 and scFvSSaA–IL-2 were detected using a biotin-conjugated rat antirat IgG2b MoAb (1 μg/mL, PharMingen) followed by FITC-labeled avidin (1:500; Cappel). Cells were then fixed in 2% paraformaldehyde/PBS for analysis on a FACS (FACSstation; Becton Dickinson, Mountain View, CA).
Cytokine proliferation assays. The biological activity of test samples was assayed by their ability to support the proliferation of a murine IL-2/IL-6 responsive T cell line, HT-2. Samples were added in triplicate to 96-well plates in RPMI-10 with 5 x 10^5 HT-2 cells to a total volume of 0.1 mL and incubated for 16 to 24 hours at 37°C, 5% CO2 in a humidified incubator. In the assays that involved membrane-bound IL-2 activity, 2 x 10^5 irradiated (2,000 rad) 38C13 cells were incubated with 10 μg/mL of test samples at 4°C for 30 minutes, followed by treatment with 1% paraformaldehyde/PBS for 10 minutes at room temperature (RT). The Ab-coated cells were then harvested and added to the HT-2 culture. After 16 hours, 1 μCi ^3H-thymidine (Amersham) in 50 μL of growth medium was added to each well, cells were harvested 4 to 6 hours later using a FilterMate (Packard, Meriden, CT) automatic cell harvester, and incorporated radioactivity was determined by a TopCount microplate scintillation counter (Packard). Recombinant murine IL-2 (PharMingen) was included in the assay as a positive control. All results are presented as the mean cpm ± standard deviation (SD).

Cytotoxicity assays. The ^125I IDr release assay was used to detect the cell-mediated cytotoxicity. The effector cells were prepared by incubating murine spleenocytes with anti-CD3 MoAb (1:100 dilution of culture supernatants of 145-2C11 hybridoma cells) for 2 days, followed by treatment with 30 to 50 U/mL of recombinant murine IL-2 in the absence of anti-CD3. 38C13 cells were labeled with ^125I IDr and incubated for 20 hours with effector cells in the presence of various anti-Id Abs. Recombinant IL-2 at doses equivalent to the fusion protein or a combination of chS5A8 and recombinant IL-2 was also included for comparison. The results are expressed as total percentage of lysis and net percentage of lysis according to the following formula:

\[
\text{Total \% Lysis} = \frac{(\text{cpm in Supernatant}) - (\text{cpm in Medium Control})}{(\text{cpm in Medium Control})} \times 100
\]

Specific % Lysis = (Total Lysis of Test Effectors) - (Total % of Lysis of Medium Control)

The spontaneous release (media alone) usually ranged from 5% to 15% of the total lysis. The standard error obtained with total lysis was usually between 0.5% and 5% of the mean. One lytic unit (LU) was defined as the number of effectors required to give 30% specific lysis for 5 x 10^8 target cells. LU per 10^6 cells was calculated by plotting a curve derived from specific lysis obtained from 3 to 4 effector-to-target (E/T) ratios.

In vivo clearance studies. Female BALB/c mice were injected intravenously (IV) via the tail vein with 25 μg of the various anti-Id Abs. For S5A8, chS5A8, and chS5A8–IL-2, serum was collected by a microcapillary tube from the tail vein at 0.25, 0.5, 1, 2, 4, 8, 12, 24, 48, and 72 hours after injection. Because of the short half-life of the scFv anti-Id–IL-2 fusion protein, blood samples were collected from the orbital plexus at 5, 10, 20, 30, 40, 50, 60, 120 and 240 minutes after injection. The specific Ab concentration in each sample was determined by ELISA for anti-Id determinant. Half-life values were determined based on the two-compartment open model, as previously described.39

Tumor challenge and Ab therapy. C3H/HeN mice received 10^4 38C13 tumor cells subcutaneously (SC). Treatment was started 1, 3, or 7 days after tumor inoculation by IV injection of 10 μg of various Abs over a period of 5 days, once a day. Treatment with a combination of chS5A8 and recombinant IL-2 was included as a control. Tumors were measured every other day, and the tumor volume (in cubic millimeters) was approximated by using the ellipsoidal formula: length (mm) x width (mm) x height (mm) x 0.52 (derived from π/6).40 The mean volume and SD of each group were calculated. Animals were observed until the SC tumors measured more than 3,000 mm^3 or until any mouse was observed to be suffering or appeared to be moribund. Animals under these conditions were euthanized humanely, according to institutional policy. Sacrifice dates were recorded, and the mean survival of each group was calculated. The statistical significance of differential findings between experimental groups of animals was determined by the Student’s t test. Findings were regarded as significant if two-tailed P values were ≤ 0.05.

RESULTS

Construction and characterization of anti-Id–IL-2 fusion proteins. Anti-Id MoAbs have been shown to be effective in vivo in the treatment of 38C13 murine B-cell lymphoma.22,23 A combined therapy consisting of anti-Id MoAbs and IL-2 was reported to be more effective than treatment with anti-Id alone.26 However, high-dose IL-2 administered systematically usually causes severe toxicity.31,12 and thus limits its clinical use. We reasoned that Ab-cytokine fusion proteins may offer an alternative approach to deliver large amounts of IL-2 to the tumor site in such a way as to minimize the toxicity. To this end, we made two forms of anti-Id–IL-2 fusion proteins: chS5A8–IL-2 consisting of a mouse-human chimeric IgG molecule with mouse IL-2 attached to each of the carboxy-terminal ends of the Ig Cγ3 domain, and scFvS5A8–IL-2 containing only the Vγ and Vδ domains covalently connected by a peptide linker (Fig 1A). We used human IgG1 isotype in this study, because it has been identified as the isotype of choice for building Abs with high avidity in complement fixation and ADCC.31,42 The chimeric chS5A8–IL-2 were produced from transfectoma and purified by affinity chromatography using protein A-Sepharose. The scFvS5A8–IL-2 fusion protein was produced in baculovirus-infected insect cells and purified by affinity chromatography prepared with 38C13 Id protein, as previously described.33 To serve as controls for chS5A8–IL-2, a mouse-human chimeric version of S5A8, designated chS5A8, and a chain-shuffle IgG IL-2 fusion protein, designated TAY–IL-2, were similarly constructed. The structure of TAY–IL-2 was identical to chS5A8–IL-2, except that its Vδ domain was derived from an unrelated Ig light chain and was expected to lose the 38C13 Id-binding activity.

The various anti-Id Abs were subjected to SDS-PAGE (Fig 1B). Under reducing conditions, the light and heavy chains of S5A8 and chS5A8 migrated at an apparent molecular weight (MW) of 29 and 31 kD and 55 and 55 kD, respectively (Fig 1B, lanes 5 and 6). Reduction of the chS5A8–IL-2 fusion protein gave a 31-kD light chain similar to that of chS5A8, but the heavy chain molecule migrated at an apparent MW of 76 kD, indicating that it contained an IL-2 tail (Fig 1B, lane 7). The heavy and light chains were properly assembled to give tetrameric proteins of MW 158 kD (S5A8 and chS5A8) and 212 kD (chS5A8–IL-2), as could be observed on a nonreducing gel (Fig 1B, lanes 1, 2, and 3). The scFvS5A8–IL-2 fusion protein was produced as a monomer with an apparent MW of 51 kD, as shown in the reducing and nonreducing gel (Fig 1B, lanes 4 and 8). Immunoblot analysis showed that all S5A8-derived anti-Id Abs retained the 38C13 Id-binding activity (Fig 1C, lanes 1 through 4), but only the chS5A8–IL-2 and scFvS5A8–IL-2 fusion proteins were recognized by the specific anti-IL-2 Ab (Fig 1C, lanes 7 and 8).

Id-binding and IL-2 biological activities of anti-Id–IL-2 fusion proteins. Competition assays were conducted to evaluate the immunoreactivity of purified anti-Id–IL-2 fusion proteins against 38C13 Id. Increasing concentrations of S5A8, chS5A8, and chS5A8–IL-2 and two monovalent Abs, scFvS5A8–IL-2 and S5A8 Fab fragment, were evaluated for
their ability to inhibit binding of biotinylated S5A8 to 38C13 Id
(Fig 2). The relative Id-binding activities of S5A8, chS5A8, and
chS5A8–IL-2 were comparable: maximum inhibition (100%)
was achieved at 5 nmol/L, whereas 50% inhibition was at 0.2 to
0.3 nmol/L. The monovalent S5A8 Fab had about threefold
lower binding activity to 38C13 Id with maximum and 50%
inhibition achieved at 17 and 0.7 nmol/L, respectively. The
scFvS5A8–IL-2 fusion protein had an even more significant
reduction in the Id-binding activity: 100% inhibition was not
achieved over the range of concentrations tested and its 50%
inhibition was at 8.7 nmol/L. Thus, the relative binding ability
of chS5A8–IL-2 was comparable to that of the parent S5A8 or
the chimeric chS5A8 anti-Id Abs; however, the scFvS5A8–IL-2
fusion protein was about 30- to 40-fold lower in its Id-binding
activity than the bivalent Abs.

The ability of anti-Id–IL-2 fusion proteins to bind to tumor
cells was examined by reacting the fusion proteins with 38C13
tumor or with V1-1, an Id-negative variant.28 Figure 3 shows
the result of flow cytometry analysis demonstrating that chS5A8–
IL-2 and scFvS5A8–IL-2 fusion proteins recognized 38C13
tumor cells equally well as the parent S5A8 or the chimeric
chS5A8 Abs (Fig 3B through E). However, none of the various
anti-Id Abs reacted with the V1-1 variant (Fig 3G through J),
indicating that the recognition of 38C13 cells was Id specific.
Experiments using an Ig-negative variant of 38C13 have shown
similar results (data not shown). Thus, both forms of the
anti-Id–IL-2 fusion proteins were specific for the 38C13 Id and
were capable of binding to the tumor cells. We also showed that
the chain-shuffle TAY–IL-2 fusion protein, containing the S5A8
VH and a nonspecific VL, did not bind to 38C13 tumor cells
(data not shown), indicating that both the heavy and light chains
of S5A8 made a contribution to the Id-binding activity.

To determine IL-2 activity of the fusion proteins, purified
proteins were analyzed for their ability to support the prolifera-
tion of an IL-2–dependent T-cell line, HT-2. The free anti-Id

Fig 1. Schematic diagram of the chimeric and scFv anti-Id–IL-2
fusion proteins. Solid and shaded areas represent light-chain and
heavy-chain variable regions from an anti-Id mAb, S5A8. Open areas
represent human \( \gamma_1 \) and \( \kappa \) constant regions. Checkered regions
represent the mature IL-2 sequence. (B) SDS-PAGE of S5A8 (lanes 1
and 5), chS5A8 (lanes 2 and 6), chS5A8–IL-2 (lanes 3 and 7), and
scFvS5A8–IL-2 (lanes 4 and 8) under nonreducing (lanes 1 through 4)
or reducing (lanes 5 through 8) conditions. The MW is determined by
marker proteins. (C) Immunoblot analysis of the various anti-Id Abs.
S5A8 (lanes 1 and 5), chS5A8 (lanes 2 and 6), chS5A8–IL-2 (lanes 3 and
7), and scFvS5A8–IL-2 (lanes 4 and 8) under nonreducing (lanes 1
through 4) or reducing (lanes 5 through 8) conditions were subjected
to SDS-PAGE followed by electroblotting to nitrocellulose. Nitrocellu-
lose strips were reacted with 38C13 Id (lanes 1 through 4) or rat
antimouse IL-2 (lanes 5 through 8) and detected with horseradish
peroxidase-conjugated second-step reagents.

Fig 2. Competition assays comparing the relative inhibition of
binding of biotinylated S5A8 by unlabeled S5A8, chS5A8, and chS5A8–
IL-2 Abs as well as two monovalent anti-Id Abs, scFvS5A8–IL-2 and
S5A8 Fab fragment. Microtiter plates were coated with purified
38C13 Id. Serial dilution of each unlabeled competitor anti-Id Abs
were added to each well together with a fixed amount of biotinylated
S5A8 and incubated overnight at 4°C. The bound biotinylated S5A8
were then quantitated. The percentage of inhibition values represent
the mean of triplicate trials. SE values were within 10% of the mean.
Abs, S5A8 and chS5A8, were completely negative in this assay. In contrast, both anti-Id–IL-2 fusion proteins clearly demonstrated the ability to stimulate the growth of HT-2 cells in a dose-dependent manner (Fig 4A). On a molar basis, chS5A8–IL-2 and scFvS5A8–IL-2 had approximately 200% and 50%, respectively, of the activity required to produce 50% maximum proliferation of HT-2 cells compared with the recombinant IL-2 standard. The chain-shuffle TAY–IL-2 fusion protein, although having lost Id-binding ability, exhibited IL-2 activities similar to the Id-specific chS5A8–IL-2 fusion protein (data not shown).

The activity of the fusion proteins could also be inhibited by the anti-IL-2 Ab, indicating that the cytokine activity was specific (data not shown). Both forms of the anti-Id–IL-2 fusion proteins were further tested for functional duality by binding to irradiated 38C13 tumor cells and then using the Ab-coated tumor cells to stimulate the proliferation of HT-2 cells. The results shown in Fig 4B clearly demonstrated that the tumor cell-bound chS5A8–IL-2 and scFvS5A8–IL-2 efficiently stimulated the growth of HT-2 cells. In contrast, S5A8- or chS5A8-coated tumor cells did not cause any significant proliferation of HT-2 cells.

Pharmacokinetic analysis of anti-Id–IL-2 fusion proteins. The pharmacokinetics of the various anti-Id Abs were examined in normal BALB/c mice after IV injection. Sera were collected at various time points after injection, and the specific Ab concentration in each sample was determined by ELISA for the anti-Id determinant. S5A8 and the mouse-human chimeric chS5A8 had similar clearance rates. The α phase (distribution of the protein from the blood to extravascular space) half-life of S5A8 was 5.6 hours and the β phase (elimination of the protein from the body) half-life was 80.2 hours, whereas α phase and β phase half-lives of chS5A8 were 3.2 and 90.5 hours, respectively (Table 1). The chS5A8–IL-2 fusion protein was more rapidly cleared from the circulation compared with the free anti-Id Abs. This was mainly due to the rapid clearance rate of the β phase (17.6 hours) of chS5A8–IL-2, because its α phase half-life (2.4 hours) was only slightly different from that of the free Abs. The scFvS5A8–IL-2 fusion protein was most rapidly cleared from the circulation among the anti-Id Abs tested, with an α phase half-life of only 5 minutes and a β phase half-life of 49 minutes.

Cell-mediated cytotoxicity is enhanced by anti-Id–IL-2 fusion proteins. The various S5A8-derived anti-Id Abs were evaluated for their ability to mediate cytolyis of 38C13 target cells using 125I UdR release assay.8 Spleen cells stimulated with anti-CD3 Ab and recombinant IL-2 were used as a source of effector cells. The cytotoxicity was expressed in LU, which was defined as the number of effectors required to give 30% specific lysis for 5 × 10⁴ target cells. At a concentration of 100 ng/mL, S5A8 and chS5A8 did not increase specific lysis of target cells compared with the medium alone control (Fig 5). In contrast, the lytic potential of CD3-activated blasts was significantly enhanced by the addition of either chS5A8–IL-2 or scFvS5A8–IL-2 fusion proteins, which was equally as effective as an equivalent amount of chS5A8 and recombinant IL-2 in potentiating tumor cell lysis. When the CD3-activated blasts were incubated with IL-2 alone, the specific lysis of tumor cells was also enhanced, indicating that the increased cytotoxicity mediated by chS5A8–IL-2 and scFvS5A8–IL-2 was mainly due to the IL-2 biological activity of the fusion proteins. The enhanced tumor cell lysis by the anti-Id–IL-2 fusion proteins was only observed with CD3- and IL-2 activated blasts but not with splenocytes without previous activation (data not shown).

Antitumor activity of anti-Id–IL-2 fusion proteins. To determine the in vivo antitumor activity of the anti-Id–IL-2 fusion proteins, syngeneic animals were inoculated SC with 3 × 10⁵ 38C13 tumor cells and treated beginning on the following day by IV administration of 10 µg per day of S5A8, chS5A8, or chS5A8–IL-2 over a period of 5 days. Mice treated with PBS alone or the nonspecific TAY–IL-2 fusion protein were included as controls. The percentage of survivors and the tumor volume progression curves are shown in Fig 6A. Compared with mice receiving PBS alone, treatment with S5A8 or chS5A8 anti-Id Abs led to tumor suppression to some extent; however, no long-term survivors were observed. Treatment with the nonspecific TAY–IL-2 fusion protein did not show any inhibition of tumor growth and resulted in only 20% long-term survivors (P > .05), which was not statistically different from PBS.
controls. In contrast, the specific chS5A8–IL-2 fusion protein produced 100% long-term survivors (P < .0001). One of the 10 mice initially exhibited a small tumor nodule that eventually regressed. In a separate experiment, the chimeric IgG and the scFv anti-Id–IL-2 fusion proteins were compared for their in vivo antitumor activity. As shown in Fig 6B, chS5A8–IL-2 significantly suppressed tumor growth and resulted in 70% long-term survivors (P < .001). Treatment with the scFvS5A8–IL-2 fusion protein did not show any inhibition of tumor growth and led to only 10% long-term survivors (P > .1). The chS5A8–IL-2 fusion protein also proved to be more proficient than a combined therapy with equivalent doses of chS5A8 and recombinant IL-2. The combined therapy was effective in delaying

Table 1. Blood Clearance Half-Life of the Free Anti-Id Abs and the Anti-Id–IL-2 Fusion Proteins

<table>
<thead>
<tr>
<th></th>
<th>t1/2α</th>
<th>t1/2β</th>
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<tbody>
<tr>
<td>S5A8</td>
<td>5.6 h</td>
<td>80.2 h</td>
</tr>
<tr>
<td>chS5A8</td>
<td>3.2 h</td>
<td>90.5 h</td>
</tr>
<tr>
<td>chS5A8–IL-2</td>
<td>2.4 h</td>
<td>17.6 h</td>
</tr>
<tr>
<td>scFvS5A8–IL-2</td>
<td>5 min</td>
<td>49 min</td>
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BALB/c mice were injected IV with 25 µg of the various anti-Id Abs and blood samples were collected at various time points after injection. The specific Ab concentration in each sample was determined by ELISA for anti-Id determinant. The α phase (distribution) and the β phase (elimination) half-life values were determined as described in Materials and Methods.

Fig 4. IL-2 bioactivity of anti-Id–IL-2 fusion proteins. (A) HT-2 cells were incubated with various concentrations of S5A8, chS5A8, chS5A8–IL-2, scFvS5A8–IL-2 or recombinant mouse IL-2. (B) Irradiated 38C13 cells were incubated with 10 µg/mL of various anti-Id Abs and fixed with paraformaldehyde. HT-2 cells were then incubated with 1 × 10⁴ or 2 × 10⁴ Ab-coated cells. Proliferation was measured by ³H-thymidine uptake 16 to 24 hours later. All results are expressed as the mean cpm incorporated ± SD of triplicate wells.

Fig 5. Effect of anti-Id–IL-2 fusion proteins on cell-mediated cytolytic activity. CD3-activated blasts were incubated with ¹²⁵I-labeled 38C13 cells with tested Abs for 20 hours. Values of specific lysis are represented in LU.

Fig 6. Effect of anti-Id–IL-2 therapy on the in vivo growth of 38C13 tumors. (A) and (B) represent data of two independent experiments. Syngeneic mice C3H/HeN (n = 10) were inoculated SC with 1 × 10³ tumor cells and then treated on the following day by IV injection of various Abs over a period of 5 days. Tumor growth was measured 3 times a week. The percentage of tumor-free animals and the mean tumor volume were calculated. SDs (bars) are only given at day 20 for clarity.
tumor growth but resulted in only 20% long-term survivors ($P > .05$).

We then performed a more stringent experiment with more established tumors to assess the power of the anti-Id–IL-2 fusion protein in the treatment of B-cell lymphoma. Syngeneic animals were similarly implanted with 38C13 tumor cells and treatment was started on days 1, 3, or 7 after tumor cell inoculation. Compared with the PBS control group, objective tumor growth suppression could be observed in all treated groups (Fig 7). By day 20, the last day before the control group required sacrifice, mice treated with chS5A8–IL-2 on days 3 and 7 after tumor cell inoculation had smaller mean tumor volumes (716 ± 332 mm$^3$ and 753 ± 310 mm$^3$, respectively), as compared with 2,706 ± 189 mm$^3$ in the control group. On day 20, the day-1–treated group had no measurable tumors in any of the mice. There were no long-term survivors in the control group as well as the day-3– and day-7–treated group, whereas mice treated with chS5A8–IL-2 on day 1 resulted in 80% long-term survivors (data not shown).

**DISCUSSION**

The use of cytokines as adjuvants has been applied in different forms of cancer therapy. Many attempts have been made to target cytokines to the tumor site to achieve their optimal biological effects. These include direct intratumor injection of cytokines$^{13,43}$ or ex vivo transfection of different cytokine genes into tumor cells for use as cancer vaccines.$^{15,16}$ Cytokine genes, including IL-2, IL-6, and interferon-γ, have also been delivered intratumorally using a particle-mediated gene gun technology and resulted in significant antitumor effects.$^{44}$ One of us (M.-H.T.), together with others, has previously reported that, by fusing a cytokine (granulocyte-macrophage colony-stimulating factor [GM-CSF], IL-2 or IL-4) to a nonimmunogenic tumor Id protein, it can be converted to a strong immunogen capable of inducing Id-specific Abs without other carrier proteins or adjuvants and of protecting recipient animals from challenge with an otherwise lethal dose of tumor cells.$^{30,31}$

In this study, we made anti-Id–IL-2 fusion proteins to use the unique targeting ability of the anti-Id Ab to direct IL-2 to the tumor site to locally boost the host immune response. Two forms of the anti-Id–IL-2 fusion proteins were constructed: an intact IgG fusion protein (chS5A8–IL-2) and an scFv fusion protein (scFvS5A8–IL-2). A monovalent scFv Ab approaches the minimum necessary structural component for antigen binding. Compared with the whole Ab molecule, a smaller scFv showed deeper penetration into tumors, faster blood clearance, and a higher ratio of tumor/normal tissues.$^{45,46}$ In addition, the monovalent structure of scFv Ab might have the advantage of avoiding antigen modulation. SDS-PAGE analysis showed that chS5A8–IL-2 was assembled into a complete Ab structure with MW of 212 kD (Fig 1). The scFvS5A8–IL-2 fusion protein was displayed as a single band of 51 kD on SDS-PAGE (Fig 1), comprising only one fourth the MW of chS5A8–IL-2. Both forms of the anti-Id–IL-2 fusion proteins retained specific recognition for the 38C13 Id and were able to bind to 38C13 but not to the Id-negative variant tumors (Fig 3).

We compared the in vivo antitumor activity of the chimeric IgG and the scFv anti-Id–IL-2 fusion proteins in syngeneic animals under conditions in which free anti-Id Abs were only minimally effective. We showed that the chS5A8–IL-2 fusion protein can effectively suppress in vivo growth of 38C13 tumor cells inoculated 1 day before treatment (Fig 6A and B). The majority of treated animals survived more than 6 months and were apparently free of residual or dormant tumor cells, as demonstrated by in vitro culture and in vivo transfer of splenocytes (data not shown). For animals with more established tumors, treatment with the chS5A8–IL-2 fusion protein was less effective, resulting in transient inhibition of tumor growth but no long-term survivors (Fig 7). We also demonstrated that chS5A8–IL-2 was more proficient in suppressing tumor growth than a combined therapy with equivalent doses of free anti-Id Ab and IL-2 (Fig 6B). Treatment with a structurally similar but nonspecific TAY–IL-2 fusion protein did not show any therapeutic effect (Fig 6A), indicating that the tumor-targeting ability of chS5A8–IL-2 was important for the observed antitumor effects. In contrast to the impressive antitumor effects accomplished by chS5A8–IL-2, treatment with scFvS5A8–IL-2 offered no protection against 38C13 challenge.

Compared with the chimeric anti-Id–IL-2 fusion protein, scFvS5A8–IL-2 was fourfold less active on a molar basis, as evidenced in a biological assay using the IL-2–dependent cell line HT-2 (Fig 4A). Tumor cells coated with the scFvS5A8–IL-2 fusion protein were capable of stimulating T-cell growth (Fig 4B), demonstrating the ability of scFvS5A8–IL-2 to activate host immunologic effector mechanisms in the tumor area. In vitro cytolytic analysis also showed that the scFvS5A8–IL-2 fusion protein was equally as effective as chS5A8–IL-2 in potentiating tumor cell lysis by CD3-activated blasts. Other scFv Ab–IL-2 fusion proteins previously reported were also shown to maintain the IL-2 biological activity.$^{47,48}$
Except for a slight variation in IL-2 activity, there were more profound distinctions between scFvS5A8–IL-2 and chS5A8–IL-2 that may explain their dramatically different antitumor effects. First of all, scFvS5A8–IL-2 was 30- to 40-fold lower in its Id-binding activity than its bivalent counterpart (Fig 2). The inferior binding activity of scFvS5A8–IL-2 was not only due to its monovalent structure but also due to its decreased Id-binding affinity. This was apparent when compared with the chemically prepared monovalent S5A8 Fab. Calculations based on concentration requirement for 50% inhibition in a competition assay demonstrated that S5A8 Fab was about 12-fold better in its Id-binding activity than the scFvS5A8–IL-2 fusion protein. The decreased Id-binding affinity of scFvS5A8–IL-2 was most likely due to the presence of the carboxy terminal IL-2 molecule that was separated from the V<sub>H</sub>-V<sub>L</sub> by a short 4-amino-acid linker and might thus interfere with the correct folding of the Ab domains. Another feature to distinguish the chimeric IgG and scFv anti-Id–IL-2 fusion proteins is their pharmacokinetic properties. The scFvS5A8–IL-2 fusion protein exhibited a very rapid plasma clearance in mice, with an α (distribution) phase half-life of 5 minutes and a β (elimination) phase half-life of 49 minutes. In contrast, chS5A8–IL-2 had pharmacokinetic properties that were greatly improved over that of scFvS5A8–IL-2, with an α phase half-life of 2.4 hours and a β phase half-life of 17.6 hours. The greater serum half-life of the chS5A8–IL-2 fusion protein compared with that of the scFv construct would be expected to help generate a higher concentration of IL-2 in the tumor microenvironment. Finally, the chS5A8–IL-2 fusion protein was able to enhance ADCC against its target melanoma metastases by mechanisms that involve B cells and asialo-GM1–positive NK cells.

In summary, we show that a chimeric IgG anti-Id–IL-2 fusion protein is more effective for the treatment of B-cell lymphoma than anti-Id Ab alone or a combination of anti-Id Ab and IL-2, whereas the scFv anti-Id–IL-2 fusion protein offered no protection against tumor challenge. We believe that the general approach of fusing a cytokine to monoclonal anti-Id Abs may be applicable to generate immunotherapeutic reagents for the treatment of B-cell malignancies.

REFERENCES


Interestingly, the effector cell population responsible for the observed antitumor responses was identified as CD8<sup>+</sup> T cells but not NK cells. Another interesting feature of Ab–IL-2 fusion proteins that may help in cancer treatment is their ability to increase tumor vascular permeability and enhance Ab uptake. Lymphotoxin (LT) and GM-CSF were also reported to be constructed as Ab-cytokine fusion proteins. The Ab–GM-CSF fusion protein was able to enhance ADCC against its target tumors, whereas the Ab-LT fusion protein has a direct cytotoxic effect on the tumor cells via the induction of apoptosis. In vivo animal studies have demonstrated that the Ab-LT fusion protein can effectively inhibit growth of disseminated melanoma metastases by mechanisms that involve B cells and asialo-GM1–positive NK cells.

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Treatment of B-Cell Lymphoma With Chimeric IgG and Single-Chain Fv Antibody–Interleukin-2 Fusion Proteins

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