

Improving the Efficacy of Antibody-Interleukin 2 Fusion Proteins by Reducing Their Interaction with Fc Receptors

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ABSTRACT

Fusion proteins between whole antibodies (Abs) and cytokines (immunocytokines) such as interleukin 2 have shown efficacy in several mouse tumor models despite a circulating half-life that is significantly shorter than that of the original Ab. We have examined the potential mechanisms responsible for clearance and shown that an important factor is enhanced binding to Fc receptor (FcR). Improvements in the half-lives of two different immunocytokines were made by changing the isotype of the human heavy chain C region from IgG1 or IgG3 to those with reduced binding to FcR, *e.g.*, IgG4. The same effect could also be achieved through site-directed mutagenesis of the FcR binding site in the IgG1 H chain. *In vitro* studies using mouse J774 FcR-expressing cells showed increased binding of interleukin 2-based immunocytokines, relative to their corresponding Abs, and that this was reversed in those fusion proteins made with IgG4 or mutated IgG1 H chains. All of the fusion proteins showing reduced FcR binding also had reduced Ab-dependent cellular cytotoxicity activity, as measured in 4-h chromium release assays. A complete loss of complement-dependent cytotoxicity activity was seen with an IgG4-based immunocytokine derived from an IgG1 Ab with potent activity. Despite these reduced effector functions, the IgG4-based immunocytokines with extended circulating half-lives showed equivalent (in the case of severe combined immunodeficiency mouse xenograft models) or better (in the case of syngeneic models) efficacy in mouse tumor models than the original IgG1-based molecules. These novel immunocytokines may show improved efficacy in therapeutic situations where T cell- rather than natural killer- or complement-mediated antitumor mechanisms are involved.

INTRODUCTION

Fusion proteins of intact Abs² and cytokines, or “immunocytokines,” have been shown to mediate the eradication of established metastatic tumors in several animal models (1–4). Virtually all of these immunocytokines have been constructed using either the human C γ 1 or C γ 3 heavy chains to preserve the potent effector functions of these isotypes: either ADCC or CDC. The underlying principle of these molecules is based on the targeting of potent cytokines to the tumor microenvironment, where they elicit an immune response that is capable of destroying the tumor. The effector cells responsible for this immune destruction have been shown (in the case of IL-2 immunocytokines) to be dependent on the tumor model: in melanoma and colon carcinoma models, the destruction is mediated by CD8⁺ T cells, whereas in a metastatic neuroblastoma model, NK cells are primarily responsible for the antitumor activity (1, 5). The importance of specific tumor targeting has also been demonstrated by control experiments with immunocytokines that do not bind the tumor but otherwise have the same biological properties of the specific immunocytokine.

These properties include an increased circulating half-life (6) and an enhanced uptake in lymph tissue (7).

What is not known is where the process of immune stimulation, mediated by the immunocytokine, occurs: at the tumor site or, potentially, in the draining lymph node. It is possible that accumulation of the fusion protein in lymph tissue focuses IL-2 activity at a site where it can make contact with circulating tumor cells as they seed the antigen-presenting cell network. Another question relates to the relative contribution of the Ab effector functions such as ADCC and CDC to the overall eradication of tumor by IL-2 immunocytokines. The complex nature of these molecules is based on their multiple biological activities and the ability to bind to multiple ligands *in vivo*. The consequences of such binding are manifested in both immunological terms as well as in basic pharmacological principles. Specifically, the binding to cell-bound ligands (receptors) can effect responses in the target cell and determine the biodistribution pattern of the protein. For example, binding to Fc and IL-2Rs would be expected to elicit immune responses and to alter the overall clearance and distribution in both the circulation and tissue compartments and, as such, would be expected to effect how much immunocytokine reaches a solid tumor.

We reported earlier that a family of Ab-cytokine proteins of the IgG1 subclass had vastly different clearance rates in mice that correlated with increased antigen binding activity, suggesting some type of alteration in Ab structure (8). A fusion protein with granulocyte macrophage colony-stimulating factor did not have increased antigen binding, relative to the original Ab, and had a long half-life that was more similar to that of the Ab than that of the cytokine. An IL-2 fusion protein, on the other hand, had a dramatic effect on antigen binding (at least with this antiganglioside Ab) and had the fastest rate of clearance from the circulation. In these studies, it was not possible to conclude that the faster clearance was due, instead, to binding through the IL-2R on cells in the circulation because human IL-2 will bind to and activate mouse IL-2R-bearing cells. Despite this relatively short half-life, compared to normal IgG, humanized whole Ab-IL-2 fusion proteins have proven to be highly efficacious in mouse tumor models (1–5).

In this study, we attempted to improve the circulating half-lives of Ab fusion proteins that are cleared more rapidly than free Abs. Our approach was to use heavy-chain isotypes as fusion partners that have reduced affinity or lack the ability to bind C1q or Fc γ R on cells or genetic modification of the residues in C γ 1 and C γ 3 H chains that are essential for such binding. The rationale was based on the possibility that fusion of a protein (in this case, IL-2) to the COOH terminus of an intact Ab altered its affinity for these ligands, thereby leading to its removal from the circulation.

We have also performed preliminary efficacy studies comparing fusion proteins with shorter or longer half-lives and corresponding reduced binding to FcR. The ability of the same set of Abs to mediate ADCC was also used to assess the effect of these changes on this potentially important effector function. As expected, alteration of the optimal FcR binding motif reduced the ability of the Ab fusion protein to mediate ADCC, presumably by reducing the affinity of cell-bound fusion protein for Fc γ RIII on NK cells. Nonetheless, the use of Ab-IL-2 fusion proteins with longer half-lives (but reduced ADCC activity *in vitro*) proved to be equally or more efficacious in several

Received 10/27/98; accepted 3/3/99.

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² The abbreviations used are: Ab, antibody; ADCC, Ab-dependent cellular cytotoxicity; CDC, complement-dependent cytotoxicity; IL-2, interleukin 2; NK, natural killer; IL-2R, IL-2 receptor; FcR, Fc receptor; FACS, fluorescence-activated cell sorter; HRP, horseradish peroxidase; SCID, severe combined immunodeficiency; LAK, lymphokine-activated killer.

metastatic tumor models, relative to the well-studied versions constructed using the human C γ 1 H chain.

MATERIALS AND METHODS

Animals and Cell Lines. BALB/c and BALB/c *scid/scid* mice were purchased from Taconic Farms (Germantown, NY). Mouse renal carcinoma (RENCA) cells were transfected by electroporation with an expression vector containing the cDNA for human EpCAM, driven by the cytomegalovirus promoter (9). Transfectants resistant to G418 were tested for cell surface expression of human EpCAM by immunostaining with KS-1/4 Ab and FACS analysis. A stable expressing clone (RENCA-EpCAM) was maintained in DMEM containing 10% fetal bovine serum and G418 (500 μ g/ml; Life Sciences Technologies Inc., Gaithersburg, MD) for several passages without loss of expression.

Recombinant Abs and Fusion Proteins. The original humanized Ab-IL-2 fusion protein (huKS-IL-2), derived from the mouse KS-1/4 Ab, has been described in detail (9). A IgG4 version of the Ab-IL-2 fusion protein was constructed by removing the C γ 1 gene fragment from the huKS-IL-2 expression vector and replacing it with the corresponding sequence from the human C γ 4 gene. A mutant form of the C γ 1 fusion protein was engineered by PCR mutagenesis using overlapping primers that change the sequence from Glu²³³-Leu²³⁴-Leu²³⁵-Gly²³⁶ (found in C γ 1) to the Pro-Val-Ala sequence of the C γ 2 isotype. The overlapping primers were used in separate PCRs, together with upstream and downstream primers that flank the *Pst*I-*Drd* I fragment extending from the beginning of the hinge exon to part way into the CH2 exon of the human C γ 1 gene. The PCR products of the individual reactions were purified on agarose gels, and aliquots of each were mixed and amplified with the outer primers in a second PCR to give the joined, mutated fragment. The resulting *Pst*I-*Drd* I fragment was used to replace the original fragment in the huKS-IL-2 (C γ 1) expression vector.

The final vectors, containing either the C γ 4-IL-2 H chain or mutated C γ 1-IL-2 replacement, were introduced into mouse myeloma cells by electroporation, and transfectants were selected by growth in medium containing methotrexate (0.1 μ M).

Cell clones expressing high levels of the Ab-IL-2 fusion protein were expanded, and the fusion protein was purified from culture supernatants using protein A-Sepharose chromatography. The purity and integrity of the fusion proteins were determined by SDS-PAGE. IL-2 activities were measured in a T-cell proliferation assay using the CTLL-2 cell line and found to be identical to that of the γ 1 construct.

FCR Binding Assays. The various Ab-IL-2 fusion proteins were individually diluted to 2 μ g/ml in PBS containing 0.1% BSA, together with 2×10^5 J774 cells in a final volume of 0.2 ml. Duplicate reactions also contained mouse IgG (50 μ g/ml) as competitor. After incubation on ice for 20 min., cells were washed twice with PBS-BSA, a FITC-conjugated antihuman IgG Fc Ab F(ab')₂ (Jackson ImmunoResearch, West Grove, PA) was added, and incubation was continued for an additional 30 min. Unbound Abs were removed by two washes with PBS-BSA, and the cells were analyzed in a FACS. Control reactions contained the same cells mixed with just the FITC-labeled secondary Ab or with the humanized KS-1/4 (γ 1) or 14.18 (γ 1) Ab (no fused IL-2).

Cell-based ELISA for binding to J774 cells used confluent monolayers seeded at 10^5 cells/well in 96-well plates and incubated overnight at 37°C. Cells were fixed with 1% formalin in PBS and rinsed with PBS. Recombinant Abs and fusion proteins were added at varying concentrations in PBS containing 0.5% BSA and incubated at room temperature for 1 h. Unbound proteins were removed by three washes with PBS. Bound Abs were detected after incubation for 1 h at 37°C with an antihuman Fc-specific, HRP-conjugated F(ab')₂ (Jackson ImmunoResearch).

Pharmacokinetic Analysis. Purified KS-1/4-IL-2 fusion proteins were buffer exchanged by diafiltration into PBS and diluted further to a concentration of \sim 100 μ g/ml. A volume of 0.2 ml (\sim 20 μ g) was injected into 6–8-week-old BALB/c mice in the tail vein using a slow push. Four mice were injected per group. At various time points, small blood samples were taken by retro-orbital bleeding from anaesthetized animals and collected in tubes containing heparin to prevent clotting. Cells were removed by centrifugation in an Eppendorf high-speed tabletop centrifuge for 5 min. The plasma was removed with a micropipettor and frozen at -70°C . The concentration of human Ab

determinants in the mouse blood was measured by ELISA. A capture Ab specific for human H and L Ab chains (Jackson ImmunoResearch) was used for capture of the fusion proteins from the diluted plasma samples. After a 2-h incubation in Ab-coated 96-well plates, the unbound material was removed by three washes with ELISA buffer (0.01% Tween 80 in PBS). A second incubation step used either the antihuman Fc Ab (for detection of both Ab and intact fusion protein) or an antihuman IL-2 Ab (PharMingen, San Diego, CA) for detection of only the intact fusion protein. Both Abs were conjugated to HRP. After a 1-h incubation, the unbound detecting Ab was removed by washing with ELISA buffer, and the amount of bound HRP was determined by incubation with substrate and measuring in a spectrophotometer. All pharmacokinetic experiments were repeated at least twice, but data from single representative experiments are shown in the figures.

Biodistribution of Radiolabeled KS-IL-2. Immunocytokines were labeled with ¹²⁵I using the Iodobead method (Pierce, Rockford, IL) and separated from unincorporated iodine using a G-25 column. The specific activities of the immunocytokines were 1.6 and 2.0 $\mu\text{Ci}/\mu\text{g}$ for the γ 1 and γ 4 forms, respectively. Both labeled proteins were tested in a nonradioactive version of the CTLL-2 proliferation assay (10) and were found to maintain their IL-2 proliferative activity. BALB/c mice (four per group) were injected s.c. (10^5 cell per injection) with CT26 tumor cells expressing human EpCAM, as indicated, and tumors were allowed to grow to \sim 100 mm³. Labeled immunocytokines (20 μCi) were injected i.v., and tumors were resected at the indicated time points. Tumor and organs were weighed and the amount of radioactivity was measured using a gamma counter. Results were expressed as relative percentage injected dose per g of tissue, after correcting for increased labeled protein in the blood pool (see figure legends).

Ab Effector Activities. ADCC was tested as described (9) using either ⁵¹Cr-labeled human PC-3 prostate carcinoma cells (for huKS-based proteins) or GD2-expressing M21 melanoma cells (for Hu14.18-based proteins). Specific lysis was measured after a 4-h incubation with the indicated number of resting human peripheral blood mononuclear cells. For determination of CDC, radioactively labeled M21 melanoma cells were transferred to wells of a 96-well plate together with dilutions of the recombinant proteins. Human plasma, diluted 1:8, was added as a source of human complement (50 μ l/well), and the plates were incubated for 1 h at 37°C. Percentage specific lysis was calculated by subtracting the background radioactivity from the experimental values, dividing by the total releasable radioactivity obtained by detergent lysis, and multiplying by 100.

SCID Mouse Melanoma Model. CB-17 SCID mice (6–8 weeks) were injected with GD2-expressing M24 melanoma cells (10^6 cells per mouse in a volume of 0.2 ml of PBS) on day 0. On the same day, human peripheral blood mononuclear cells were purified from a plasmapheresis of a normal healthy donor by Ficoll gradient centrifugation. The buffy coat containing mononuclear cells was isolated and cultured in RPMI 1640 containing 10% fetal bovine serum and 500 units of human IL-2 for 3 days. Prior to injection, the activated LAK cells were washed twice with PBS by centrifugation and resuspension. On the morning of day 3, tumor-bearing mice were injected in the peritoneal cavity with 10^7 LAK cells. One group of 8 mice did not receive LAK cells. Treatment with vehicle (PBS) or the indicated proteins began \sim 3–4 h later on day 3 and was repeated every day for a total of 7 days.

Mice were monitored for symptoms and were sacrificed when the control group became moribund (approximately day 21 after tumor implantation). Mice were euthanized by asphyxiation with CO₂, and the lungs were dissected, rinsed with PBS, and stained to visualize the metastases. Animals with high tumor load had fused metastatic nodules that were difficult to count. Instead of counting individual foci, the percentage of surface coverage was used for evaluation.

Syngeneic CT26-EpCAM Tumor Model. Mouse CT26 carcinoma cells expressing human EpCAM (9) were injected s.c. in the shaved backs of BALB/c mice (10^5 per injection). Five days later, mice were injected i.v. with PBS vehicle or huKS-IL-2 immunocytokine (either γ 1 or γ 4 H chain isotype) and on successive days for a total of five injections. Tumor volumes were measured with calipers and calculated as $V = 4\pi/3(0.5l \times 0.5w \times 0.5h)$, where l is the length, w is the width, and h is the height of the tumor.

Syngeneic RENCA-EpCAM Pulmonary Metastasis Model. BALB/c mice (8–10 weeks old) were injected in the tail vein with 10^5 RENCA-Ep cells in PBS. Treatment with huKS-IL-2 immunocytokines began 3 days later using daily i.v. injection for 5 consecutive days. Control and treated animals were

monitored for symptoms and sacrificed as described above. The number of lung metastases was enumerated using a dissecting microscope.

RESULTS

Pharmacokinetics of Immunocytokines of the C γ 4 Isotype. We tested the effect of H chain isotype switching in two well-characterized IL-2 immunocytokines for the purpose of seeing whether this affected the circulating half-life and antitumor activity. One of the immunocytokines was based on the anti-GD2 Ab 14.18 (reactive with neuroblastoma, melanoma, and other tumors derived from the neuroectoderm) and was originally constructed as a chimeric Ab fusion protein with a H chain of the C γ 1 isotype (6). The second immunocytokine was derived from the mouse anti-EpCAM monoclonal Ab, KS-1/4, and was constructed using humanized V regions and the human C γ 1 H chain (9). This immunocytokine reacts with virtually all human carcinomas. We chose to test our hypothesis in the two Ab systems because both immunocytokines are currently in clinical development, and we wanted to ensure that our results are not specific for one Ab system.

In our preliminary experiments, an IL-2-based immunocytokine, constructed with a C γ 4, rather than a C γ 1 human H chain C region, was administered i.v. into BALB/c mice. The pharmacokinetics of C γ 1 and C γ 4 versions of the huKS-IL-2 immunocytokine in the circulation were compared using ELISA methods that distinguish between the Ab and intact fusion protein components. This allows us to determine what amount of intact immunocytokine contributes to the measurement and how much results from proteolytic cleavage and release of free Ab in the circulation. The data in Fig. 1 show the typical pattern seen with C γ 1-based IL-2 immunocytokines. A relatively large portion of the immunocytokine was distributed out of the circulation in the first 2 h and the remainder clears at a slower rate thereafter. It is also apparent that, at later time points, a significant amount of the C γ 1 immunocytokine was proteolytically cleaved. In marked contrast, the distribution of the C γ 4-based huKS-IL-2 out of the circulation was much less, and therefore, the circulating levels remained significantly higher for many hours. Furthermore, a comparison of intact immunocytokine and total Ab components shows that very little proteolysis of the material in the blood has occurred.

Binding of Immunocytokines to FcR-bearing J774 Cells. Because IgG4 is known to bind with a 10-fold lower affinity to Fc γ RI

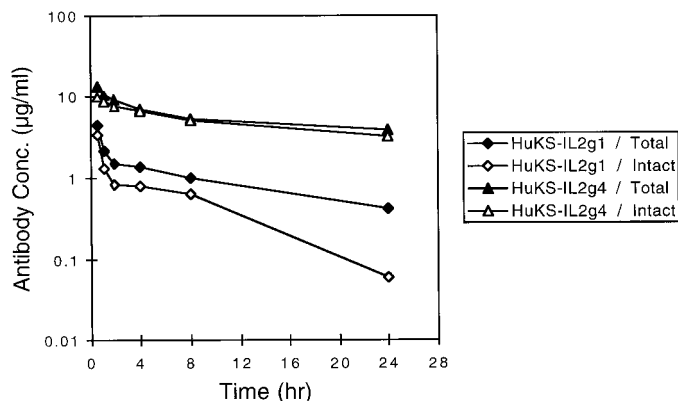


Fig. 1. Pharmacokinetics of huKS-IL-2 immunocytokines in BALB/c mice. Groups of four mice were injected i.v. with 25 μ g of either the C γ 1-IL-2 or C γ 4-IL-2 form of huKS-IL-2, and blood samples were collected at the indicated time points. The levels of total Ab (immunocytokine and free Ab) and intact immunocytokine were determined by ELISA using antihuman Fc and antihuman IL-2 Abs, respectively, for detection. Data from a representative experiment are shown. SDs between individual mice at a given time point were <20% of the experimental values. Differences between the C γ 1-IL-2 and C γ 4-IL-2 forms were significant ($P < 0.05$) for the starting time point (15 min) and were highly significant thereafter ($P < 0.01$).

than IgG1, we tested whether binding to this cellular receptor correlated to the dramatic change in circulating $t_{1/2}$ of the IL-2 immunocytokines. Binding of the huKS Ab, huKS γ 1-IL-2, and huKS γ 4-IL-2 proteins to the mouse J774 macrophage cell line was measured by detecting the cell-bound protein with an antihuman Fc FITC conjugate (Fab' dimer) and FACS analysis. Results (Fig. 2A) show that a much higher degree of binding occurred with the C γ 1-IL-2 immunocytokine than with the huKS Ab (also a C γ 1 isotype) or the C γ 4-IL-2 immunocytokine and that most of this binding could be competed with excess mouse IgG. This rules out the possibility that the binding was mediated through other receptors, e.g., IL-2R.

We also compared the ability of three isotypic forms of the huKS-IL-2 immunocytokine to bind to plate-bound J774 cells using and ELISA based assay. Both the C γ 1 and C γ 3 forms bound strongly to J774 cells, whereas the C γ 4 form was \sim 10-fold less effective (Fig. 2B). These results are not limited to just the huKS Ab system because similar results were obtained in a direct binding assay when we compared the chimeric anti-GD2 ganglioside Ab, ch14.18, and ch14.18-IL-2 immunocytokine as well as humanized forms of the Ab and C γ 1 and C γ 4 IL-2 immunocytokines (Fig. 2C). Clearly, the fusion of IL-2 to these Abs has increased their affinity to FcR-bearing cells, and this increased binding can be reduced by sequence changes in the H chain known to be responsible for FcR binding. Alterations in binding to GD2 antigen due to fusion of IL-2 were reported earlier (6, 8) and are likely the consequence of the same process.

Mutational Analysis of Altered FcR Binding and Pharmacokinetics. Both human IgG1 and IgG3 bind with high affinity to Fc γ RI, IgG4 binds 10-fold less well, and IgG2 does not bind at all (11). This has been correlated most strongly with the short stretch of amino acid residues (amino acids 234–237) in the CH2 domain of those Abs with high affinity (12). The C γ 4 chain has a single replacement of Phe²³⁵ for Leu²³⁵ in this motif but has an additional change at Pro³³¹ that also contributes to inefficient Fc γ RI binding as well as a lack of complement-fixing activity.

To discriminate between FcR and C1q binding as mechanisms for rapid clearance, we substituted the more drastically altered C γ 2 hinge-proximal segment into the C γ 1 H chain, which should affect FcR binding but not complement fixation. This was achieved by cloning and mutating the small region between the hinge and the beginning of the CH2 exon of the germ-line C γ 1 gene using overlapping PCRs. The mutant protein was expressed in transfected myeloma cells and purified by affinity chromatography. J774 binding studies confirmed reduced affinity of the C γ 1 mutant to mouse Fc γ RI that was similar to the C γ 4-IL-2 immunocytokine (data not shown), a result that is consistent with its reduced ADCC activity (see below).

The pharmacokinetic behavior of the C γ 1-IL-2 mutant fusion protein was compared to the original C γ 1-IL-2 and C γ 4-IL-2 fusion proteins in BALB/c mice, as described above. Results, shown in Fig. 3, show that mutating the binding site for FcR has a dramatic effect on the overall clearance rate of the Ab-IL-2 fusion proteins. The amount of intact fusion protein still remaining in the circulation at all time points was significantly higher for both the C γ 1-IL-2 mutant and C γ 4-IL-2 constructs and was significantly higher than that in the original C γ 1-IL-2 construct. These data support the conclusion that the specific reduction of binding to FcR is responsible for the increased circulating half-life of the C γ 1 mutant and C γ 4 immunocytokines and is reflected by a lack of distribution out of the circulation at the early time points. Still, we cannot rule out an additional role for complement binding mediated through the Pro³³¹-to-Ser³³¹ change in the C γ 4 immunocytokine and its possible effect on circulating $t_{1/2}$.

Effector Functions of IL-2 Immunocytokine Isotypes. The Ab isotype has been shown to play an important role in the effector functions of ADCC and CDC (13). Previously, we have shown that

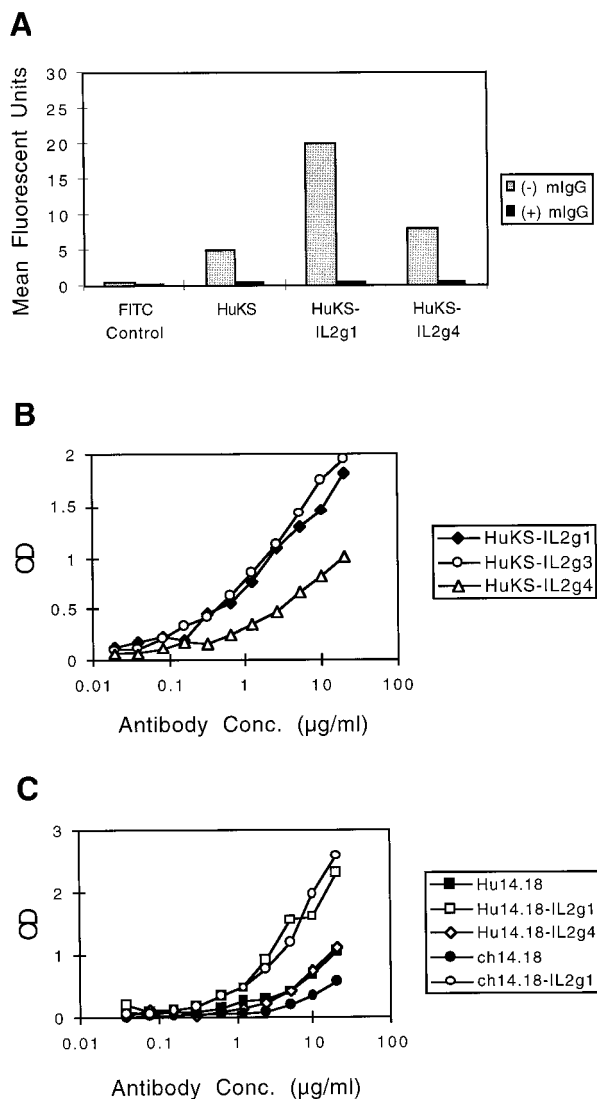


Fig. 2. Binding of isotype variant forms of immunocytokines to J774 cells. A, immunocytokine binding to mouse J774 cells in suspension was analyzed by FACS after incubation with Ab or the indicated immunocytokine (2 $\mu\text{g}/\text{ml}$), either alone or mixed with mouse IgG (50 $\mu\text{g}/\text{ml}$) as competitor. B, binding to J774 cells in 96-well plates was measured in an ELISA format using varying concentrations of three different isotypic variants of huKS-IL-2. Bound proteins were detected with an antihuman Fc-specific HRP-conjugated antiserum. C, the same assay was used to test binding of chimeric and humanized forms of the 14.18-IL-2 immunocytokine and free Ab. SDs for all of the binding experiments were $<5\%$ of the experimental values.

IL-2 immunocytokines have equal or higher ADCC activity than their Ab counterparts in short-term (4-h) cytotoxicity assays, whereas CDC activity is generally reduced (6). We tested all of the various isotypic and mutant forms of the huKS-IL-2 for ADCC activity against human PC-3 prostate carcinoma cells to confirm that reductions in FcR binding translate into reduced ADCC activity.

Results (Fig. 4A) show that fusion of IL-2 to the $\text{C}\gamma 1$ isotype of huKS Ab increased ADCC significantly, whereas both the mutant $\text{C}\gamma 1$ and $\text{C}\gamma 4$ forms had little activity, consistent with their reduced FcR binding properties. In fact, the increased ADCC activity of the $\text{C}\gamma 1$ isotype may be due to the increased affinity for FcR rather than the presence of IL-2 in this short-term assay. The $\text{C}\gamma 3$ -IL-2 immunocytokine that showed strong binding to J774 cell FcR, had little or no ADCC activity in this assay. This may reflect differential binding of the $\text{C}\gamma 3$ -IL-2 protein to the $\text{Fc}\gamma\text{RI}$ form on J774 cells and the $\text{Fc}\gamma\text{RIII}$ (CD16) form expressed on the human NK cell effectors responsible for most of the ADCC activity.

Similar analyses were performed with the various forms of the anti-GD2 Ab, 14.18, in both ADCC and CDC assays using human melanoma line M21 as the target (Fig. 4B). Again, there was a significant increase in ADCC activity between the Hu14.18 Ab ($\text{C}\gamma 1$ isotype) and the $\text{C}\gamma 1$ -IL-2 immunocytokines (humanized and chimeric), whereas the $\text{C}\gamma 4$ -IL-2 form showed a 30–50-fold reduction in ADCC activity, relative to the $\text{C}\gamma 1$ -IL-2 form.

The same set of 14.18 fusion proteins and appropriate target cells were used to measure CDC activity, using human plasma as a source of complement. As expected from our earlier studies, the IL-2 immunocytokines (both $\text{C}\gamma 1$ isotypes) had reduced CDC activities, relative to the humanized or chimeric 14.18 Abs (Fig. 4C). Also as expected from the known properties of IgG4, the $\text{C}\gamma 4$ -IL-2 immunocytokine had no detectable CDC activity. Therefore, the changes made in both of these Abs to increase their circulating half-lives have greatly diminished or removed their effector functions of ADCC and CDC.

Biodistribution of $\text{C}\gamma 4$ -IL-2 Immunocytokines. The increased circulating $t_{1/2}$ of the $\text{C}\gamma 4$ -IL-2 immunocytokine over extended times suggests that the biodistribution pattern might be significantly different from the $\text{C}\gamma 1$ -IL-2 form. In fact, if this effect is due to reduced binding to FcR, there should be reduced localization to organs containing high levels of the receptor and increased localization to antigen-expressing tissue (*i.e.*, tumor). To test this hypothesis, we labeled both the $\text{C}\gamma 1$ and $\text{C}\gamma 4$ forms with ^{125}I and injected them into mice

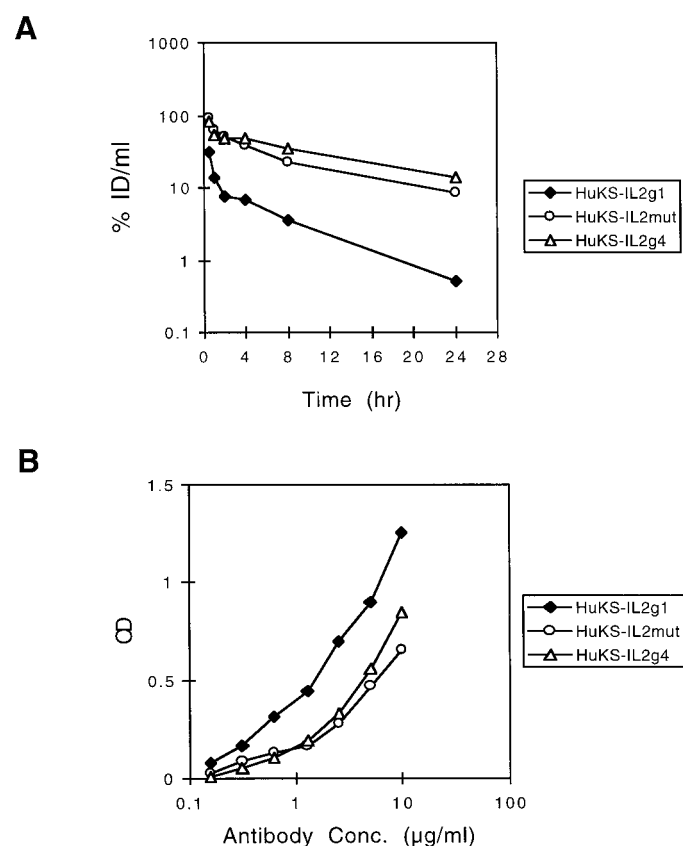


Fig. 3. Pharmacokinetics of mutated huKS-IL-2 in BALB/c mice. A, groups of four mice were injected i.v. with 25 μg of $\text{C}\gamma 1$ -IL-2, $\text{C}\gamma 1$ -IL-2 mutated to decrease FcR binding, or $\text{C}\gamma 4$ -IL-2 form of huKS-IL-2, and blood samples were collected at the indicated time points. The levels of intact immunocytokine were determined by ELISA, and the data were expressed as percentage injected dose/ml based on initial ELISA values (5 min after injection). This gave more accurate results than absolute ELISA values due to variations in immune reactivity of the three proteins. Differences between either the mutated $\text{C}\gamma 1$ -IL-2 or $\text{C}\gamma 4$ -IL-2 forms and the original $\text{C}\gamma 1$ -IL-2 form were significant ($P < 0.01$), whereas those between the $\text{C}\gamma 4$ -IL-2 and mutated $\text{C}\gamma 1$ -IL-2 forms were not. B, binding of the mutant $\text{C}\gamma 1$ -IL-2 form to J774 cells was performed as in Fig. 2.

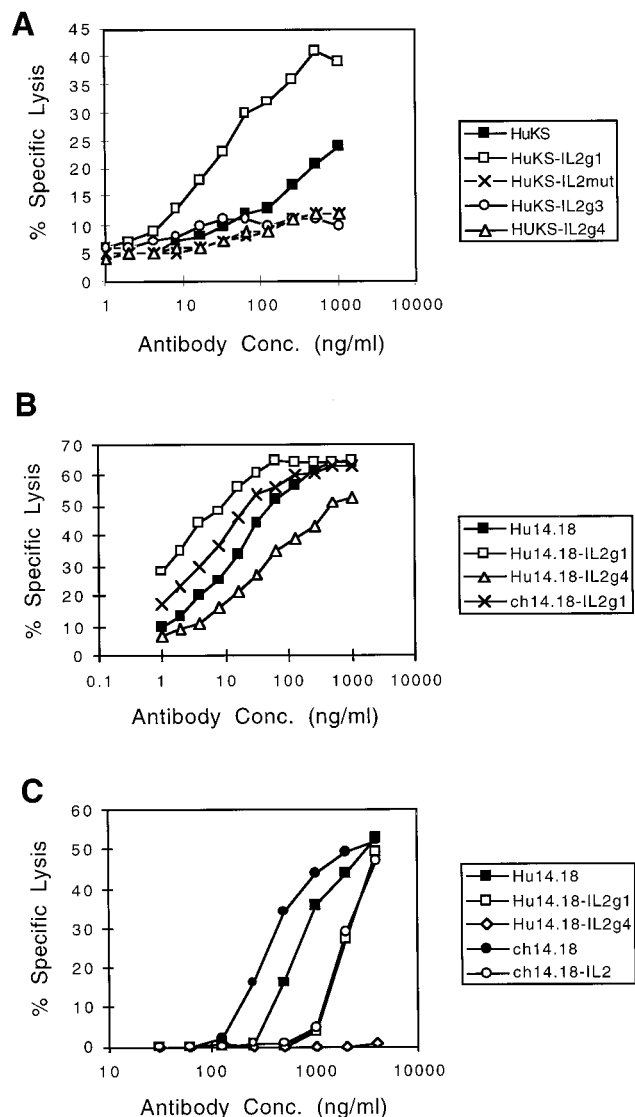


Fig. 4. Ab effector activities of immunocytokine isotype variants. A, huKS-1/4 Ab and various forms of IL-2 immunocytokines were tested in a 4-h ^{51}Cr release assay using PC-3 human prostate carcinoma cells as targets. B, humanized or chimeric forms of 14.18-IL-2 were tested in a 4 h assay using M21 human melanoma cells as targets. C, humanized and chimeric forms of 14.18 and 14.18 IL-2 were tested for CDC of M21 melanoma cells in a 1-h ^{51}Cr release assay using human complement. Differences between the $\text{C}\gamma 1$ -IL-2 and $\text{C}\gamma 4$ -IL-2 forms of both the KS and 14.18 immunocytokines were highly significant ($P < 0.01$).

bearing human EpCAM-expressing CT26 colon carcinoma skin tumors.

Results show an enhancement of uptake into EpCAM-expressing tumors with the huKS $\gamma 4$ -IL-2 immunocytokine, relative to the $\text{C}\gamma 1$ form (Fig. 5A). The percentage increase was corrected for the increase in counts in the blood pool due to the longer circulating $t_{1/2}$ of the $\text{C}\gamma 4$ -IL-2 form (68% increase; Fig. 5A). Gel analysis of the integrity of the two radiolabeled immunocytokines at the time of the distribution analysis (4 h) showed them to be intact (little or no cleavage of IL-2), whereas significant cleavage of the $\text{C}\gamma 1$ -IL-2 form had occurred at the 24-h time point (Fig. 5B). Much less of the $\text{C}\gamma 4$ -IL-2 immunocytokine was cleaved at this time, and the total amount of intact H chain-IL-2 fusion protein was at least 10 times higher than the $\text{C}\gamma 1$ -IL-2 form. In fact, the differences in the amounts of total and intact labeled immunocytokine at the 24-h time point closely matched the amount determined by ELISA. The 68% increase of the iodinated

$\text{C}\gamma 4$ -IL-2 form at 4 h was less than what would be predicted from the ELISA results (unlabeled proteins), which indicate at least a 3–5-fold difference between them. One explanation is that iodination of the protein alters the rate of clearance of the $\text{C}\gamma 4$ -IL-2 form during the initial distribution phase by increasing FcR binding. In fact, control experiments with iodinated huKS $\gamma 4$ -IL-2 show that it binds with higher affinity than the unlabeled control to J774 cells but does not bind as well as the $\text{C}\gamma 1$ -IL-2 form. This property is also reflected in more rapid blood clearance that is intermediate between the unlabeled $\text{C}\gamma 1$ -IL-2 and $\text{C}\gamma 4$ -IL-2 forms (data not shown). These effects of iodination were not seen with the $\text{C}\gamma 1$ -IL-2 immunocytokine.

Despite the reduction in the differences in FcR binding between the two immunocytokines, there was still a demonstrable increase in uptake of the $\text{C}\gamma 4$ -IL-2 form into tumor at the 4 h time point that likely underrepresents the true differences between the isotypes. The significant increase of radioactivity in tumor was accompanied by a dramatic reduction in the amount of radioactivity in the liver (Fig.

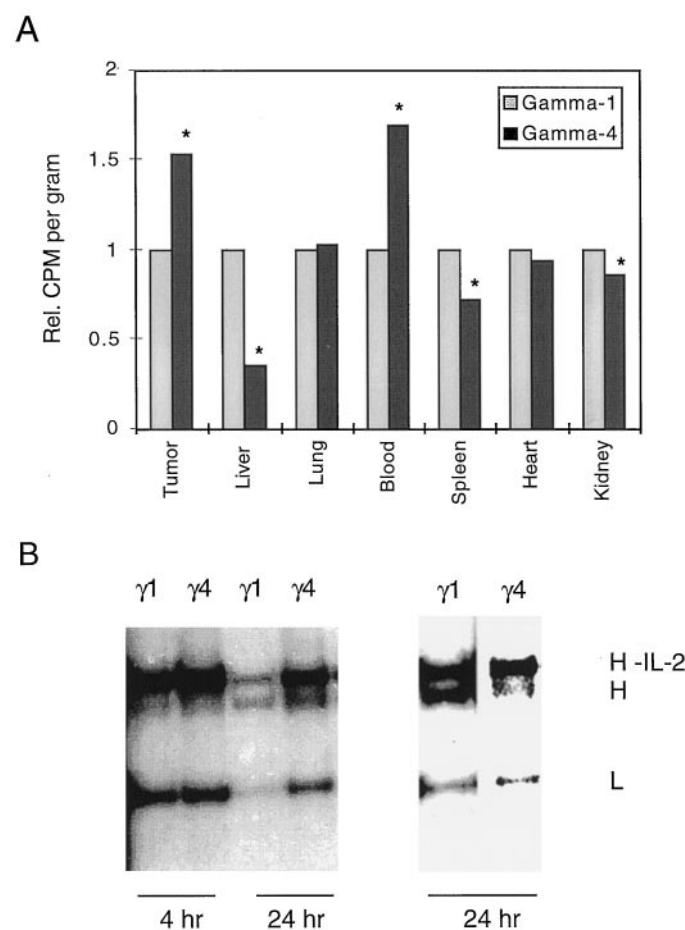


Fig. 5. Biodistribution of labeled huKS-IL-2 isotypes in mice bearing s.c. CT26-EpCAM tumors. Iodinated immunocytokines retained their IL-2 bioactivity. They were also compared in an antigen binding assay using 96-well plates coated with recombinant human EpCAM (9) and found to have the same binding activity as the unlabeled proteins. A, tumor-bearing BALB/c mice (four per group) were injected with ^{125}I -labeled $\text{C}\gamma 1$ -IL-2 or $\text{C}\gamma 4$ -IL-2 forms of huKS-IL-2 by i.v. Four h later, the mice were sacrificed and tissues were weighed and counted for radioactivity. The results were adjusted for the increased amount of the $\text{C}\gamma 4$ -IL-2 form in the blood pool by dividing experimental values by 1.68 from mice injected with this isotype. Relative values of percentage injected dose per g were calculated by dividing the experimental values by the corresponding values obtained with the $\text{C}\gamma 1$ -IL-2 form and multiplying by 100. *, statistically significant differences ($P < 0.05$). B, blood samples (50 μl) from the 4- and 24-h time points were used to isolate the immunocytokines from serum using binding to and elution from protein A-Sepharose. The eluted proteins were analyzed by SDS-PAGE after reduction with 2-mercaptoethanol. The two right lanes show long (for $\gamma 1$) and short (for $\gamma 4$) exposure times to give a more accurate representation of fusion protein integrity in each sample.

5A), a major source of FcR-bearing cells. A reduction was also seen in the amount of labeled huKS γ 4-IL-2 targeted to the spleen, another organ containing large numbers of FcR-bearing cells. Most other organs were neutral with respect to the uptake of the two immunocytokines, although a slight but significant difference was observed in the kidney.

Relative Efficacy of C γ 1-IL-2 and C γ 4-IL-2 Immunocytokines in a Xenograft Model of Human Melanoma. A SCID model was used to test the effect of reduced effector function but increased circulating half-life on the efficacy of the Hu14.18-IL-2 immunocytokine. Human effector cells (LAK cells) were transplanted into mice 3 days after tumor implantation, followed by daily treatment with either the C γ 1-IL-2 or C γ 4-IL-2 immunocytokine. Although the transplanted human LAK cells consist of both T cells and NK cells, the killing of tumor cells would likely be mediated through non-MHC-restricted mechanisms, such as direct NK cell target lysis and ADCC. Although both of these can be stimulated with IL-2, only the C γ 1-IL-2 immunocytokine would be expected to mediate ADCC.

Surprisingly, there was no difference between the efficacy of the two immunocytokines in this model (Table 1), despite the drastic reduction of ADCC and CDC activity of the C γ 4-IL-2 version. Because it is expected that tumor targeting with the C γ 4-IL-2 was enhanced over the C γ 1-IL-2 form, this may compensate for the reduction in ADCC activity. Alternatively, the local activation of NK cells may kill tumor cells independent of the ADCC mechanism, *e.g.*, through secretion of IFN- γ and activation of macrophage.

Relative Efficacy of C γ 1-IL-2 and C γ 4-IL-2 Immunocytokines in Syngeneic Carcinoma Therapy Models. Direct tumor targeting may be more important in a T cell-dependent tumor therapy model than one that is at least partially dependent on the ADCC and CDC activities of the Ab component of the immunocytokine. This is because most, if not all, activity would be due to the localized IL-2 effect. To test this hypothesis, we injected BALB/c immunocompetent mice *s.c.* with CT26 expressing human EpCAM (9). The level of surface expression of the human protein is typical of human carcinomas and does not appear to make these cells less tumorigenic. Earlier studies have shown that eradication of metastases, induced by this cell line, is dependent on CD8⁺ T cells (4). The mice were treated after tumors became palpable (5 days after implantation) with either the C γ 1-IL-2 or C γ 4-IL-2 immunocytokines for a total of 5 days. Tumor volumes were measured and compared to untreated control animals. The results clearly show a difference in the efficacy between the two forms using this treatment protocol (Fig. 6), where only two of eight mice were tumor-free at the end of the experiment following treatment with the C γ 1-IL-2 immunocytokine. Results between the C γ 1-IL-2

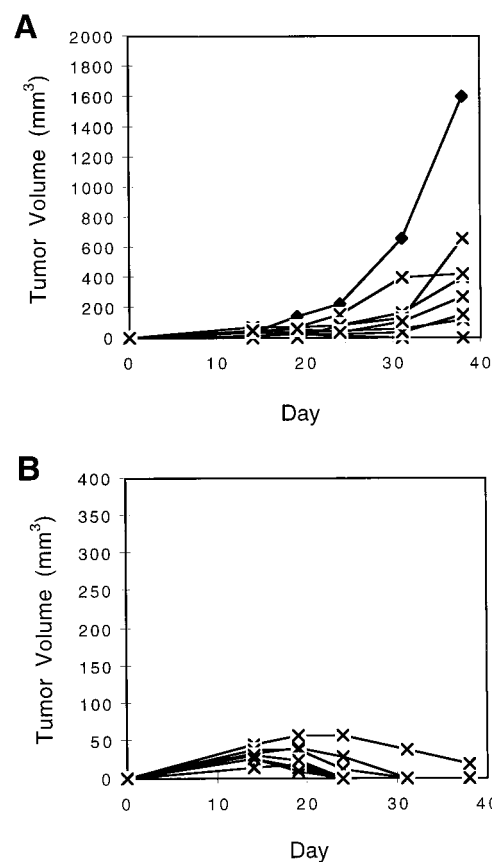


Fig. 6. Treatment of *s.c.* CT26-EpCAM tumors with huKS-IL-2. BALB/c mice were injected *s.c.* with 10^5 CT26-EpCAM tumor cells. When tumors became palpable (day 5), they were treated with 5 daily injections of either the C γ 1-IL-2 (A) or C γ 4-IL-2 (B) form of huKS-IL-2 (10 μ g/dose). X, individual mouse tumor volumes; \blacklozenge , the growth of tumor in untreated mice (PBS only). Different scales were used for the two sets of data to show that some growth and regression occurred in B.

and C γ 4-IL-2 treatment groups were significant ($P < 0.05$) at the 24-day time point and thereafter. Seven of eight mice treated with the C γ 4-IL-2 form were free of tumor at the end of the experiment and the eighth was in the process of regressing.

A second syngeneic model was used to confirm these results, but in this case, we treated established pulmonary lung metastases of the renal carcinoma line, RENCA, expressing human EpCAM. BALB/c mice bearing 3-day established metastases were treated with two doses of either the C γ 1-IL-2 or C γ 4-IL-2 immunocytokine for a short course of therapy. The shorter 3-day dosing schedule is meant to accentuate any differences between the molecules based on longer $t_{1/2}$ and improved tumor targeting. Again, the results show a significant difference in efficacy between the C γ 1-IL-2 and C γ 4-IL-2 treatment groups based on the number of countable metastases on the surface of the lungs (Fig. 7). Additional assessments of tumor efficacy based on measurements of lung weights, as well as histological examination of sectioned lung tissue, confirmed this result (data not shown).

DISCUSSION

Many factors contribute to the half-life of proteins in the circulation. With Abs, an unusually long survival time has been associated with a very slow rate of catabolism. To explain this phenomenon, Brambell *et al.* (14) postulated the existence of a protection receptor (FcRp) that would bind to the Fc portion of Abs and, following their endocytosis into cells, would redirect them back into the circulation. Only recently has it been established that a β_2 -microglobulin-contain-

Table 1 Efficacy of C γ 1- or C γ 4-based Hu14.18-IL-2 immunocytokines in a xenograft model of human melanoma pulmonary metastases

Treatment group ^a	Metastatic score ^b		Lung weights (g)	
	IgG1	IgG4	IgG1	IgG4
PBS	4,4,4,4		0.38 \pm 0.061	
Hu14.18 (16 μ g) + IL-2	4, 4, 4, 3	4, 4, 4, *	0.27 \pm 0.061	0.29 \pm 0.089
Hu14.18-IL2 (16 μ g)	0, 0, 0, 0	0, 0, 0, 0	0.17 \pm 0.008	0.18 \pm 0.002
Hu14.18-IL2 (8 μ g)	3, 2, 2, 1	2, 2, 2, 1	0.20 \pm 0.041	0.19 \pm 0.015
Hu14.18-IL2 (2 μ g)	4, 4, 4, 2	4, 4, 4, 4	0.26 \pm 0.04	0.27 \pm 0.041

^a SCID mice with established pulmonary metastases, 3 days after *i.v.* injection with 1×10^6 M24 human melanoma cells, were injected *i.p.* with 1×10^7 LAK cells followed 3 h later by the first of seven daily injections of the indicated amounts of Ab, immunocytokine, and IL-2 (48,000 IU per day). On day 28 after tumor implantation, all mice were sacrificed and assessed for tumor outgrowth by metastatic scoring and by measuring lung weights.

^b The scoring is based on the percentage surface coverage: 1, 1–25% coverage; 2, 26–50% coverage; 3, 51–75% coverage; and 4, 76–100% coverage. *, animal that died before the end of the experiment. Differences between the 8- and 16- μ g dose groups of both immunocytokines were significant ($P < 0.05$) compared to both the PBS and Ab plus IL-2 control groups.

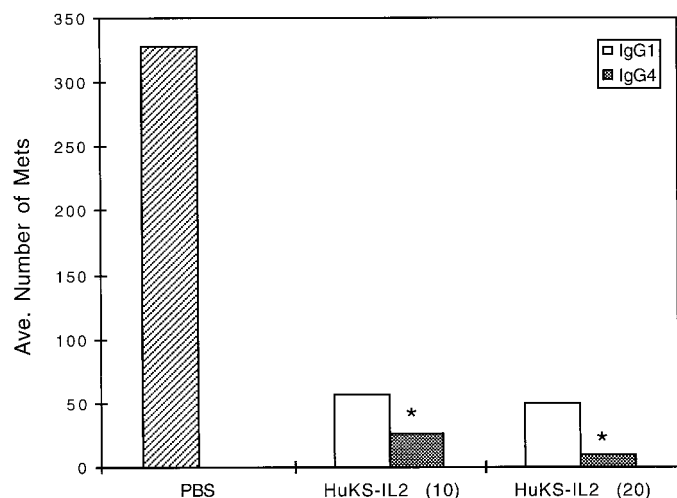


Fig. 7. Treatment of established (3-day) RENCA-EpCAM pulmonary metastases with huKS-IL-2. BALB/c mice (five per group) were treated for 5 days with 10 or 20 μg of either the C γ 1-IL-2 or C γ 4-IL-2 forms of huKS-IL-2. Surface metastases were enumerated on day 28. *, significant differences ($P < 0.05$).

ing neonatal intestinal transport receptor (FcRn) is the same as the postulated FcRp (15). The sequences necessary for binding to this receptor are conserved in all four classes of human IgG and are located at the interface between the CH2 and CH3 domains (16). In this study, we have not addressed the possible role of this class of receptor that normally has a positive effect on circulating Ab levels.

IgG molecules interact with several other molecules in the circulation including complement proteins, *e.g.*, C1q, as well as the three classes of cell-associated FcRs specific for the IgG class of Ab (Fc γ RI, Fc γ RII, and Fc γ RIII; reviewed in ref. 11). Binding of Ab to the high affinity Fc γ RI receptor occurs in the monomeric form, and thus, this receptor might have a particular ability to take up and reduce the circulating concentration of a particular Ab or Ab fusion protein.

The effect of Ab isotype on pharmacokinetic properties has been reported, and the results suggested that an Ab-IL-2 fusion protein, made using the C γ 3 rather than the C γ 1 heavy-chain constant region, had a longer half-life in animals (7). However, a direct comparison of both classes of unlabeled Ab IL-2 fusion proteins under the same experimental conditions later proved that they had similar circulating half-lives (9). This result is not surprising because the biological properties of the human IgG1 and IgG3 are quite similar and, with the exception of an extended hinge segment in IgG3, the amino acid sequence homology between them is very high.

The important sequences for Fc γ R binding are Leu-Leu-Gly-Gly (residues 234–237 in C γ 1), located in the CH2 domain adjacent to the hinge (12). These sequence motifs are conserved in C γ 1 and C γ 3, in agreement with their similar biological properties, and possibly related to the similarity of pharmacokinetic behavior when they are used to construct IL-2 fusion proteins.

In this study, we attempted to improve the circulating half-lives of Ab fusion proteins that are cleared more rapidly than free Abs. Our approach was to use heavy-chain isotypes as fusion partners that have reduced affinity or lack the ability to bind C1q or Fc γ R on cells or by genetic modification of the residues in C γ 1 and C γ 3 H chains that are essential for such binding. The rationale was based on the possibility that fusion of a protein (in this case, IL-2) to the COOH terminus of an intact Ab altered its affinity for these ligands, thereby leading to its removal from the circulation.

A comparison of fusion protein binding to mouse J774 cells, expressing Fc γ RI, showed that the fusion of IL-2 to the COOH terminus of an intact Ab does, indeed, increase its binding, relative to

the intact Ab. Immunocytokine binding to J774 cells was reduced by changing the H chain isotype from C γ 1 or C γ 3 (high-affinity binders) to C γ 4 or through mutagenesis of the FcR binding site in the C γ 1 H chain (lower-affinity binders). Pharmacokinetic studies show an inverse correlation between the affinity of the fusion protein for Fc γ RI and its half-life in the circulation. More importantly, they show a dramatic change in the amount of immunocytokine cleared immediately after injection, strongly suggesting a change in affinity for a tissue responsible for this early-phase distribution phenomenon.

The conclusion that IL-2-based immunocytokines are cleared from the circulation faster than Abs through enhanced interaction with FcR-expressing cells was supported further by measuring the clearance and biodistribution pattern of iodinated C γ 1-IL-2 and C γ 4-IL-2 forms. The data at the 4-h time point clearly show a reduction in accumulation in organs known to express high levels of Fc γ RI (liver and spleen) with the C γ 4-IL-2 immunocytokine. Because IgG4 binding to FcRII is not significantly different from IgG1 (11), it is likely that the C γ 4-IL-2 form might still bind to Fc γ RII-expressing cells in the spleen, thus explaining the less dramatic reduction in accumulation than that observed in the liver.

Finally, we have performed preliminary efficacy studies comparing fusion proteins with shorter or longer half-lives and corresponding reduced binding to FcR. The ability of the same set of Abs to mediate ADCC was also used to assess the effect of these changes on this potentially important effector function. As expected, alteration of the optimal FcR binding motif reduced the ability of the Ab fusion protein to mediate ADCC, presumably by reducing the affinity of cell-bound fusion protein for Fc γ RIII on NK cells. Nonetheless, the use of IL-2 based immunocytokines with longer half-lives (but reduced ADCC activity *in vitro*) proved to be more efficacious in two syngeneic tumor models, relative to the well-studied versions constructed using the human C γ 1 H chain. The results were particularly impressive in the treatment of 5-day established solid tumors in the skin where the differences in the half-life appears to have made more of an impact on efficacy based on increased tumor accumulation. This suggests that in these models, the longer half-life and/or the improved tumor targeting increases the effective dose of IL-2 in the tumor microenvironment and that Ab effector function plays a less important role.

In a tumor xenograft model, where most of the tumor cell killing is expected to be mediated through NK cells, the C γ 4-IL-2 immunocytokine had equivalent but not improved efficacy, despite the longer $t_{1/2}$ and increased tumor targeting. This finding suggests that there is some loss of efficacy as a result of reduced effector function but that it is compensated by the improved localization to tumor. It is also possible that there is an increase in direct NK activation that compensates for the loss of effector function. This would mean that the antitumor activity is mediated through a different mechanism. Alternatively, the C γ 4-IL-2 immunocytokine could activate a different effector cell population such as macrophage through localized IL-2 stimulation.

In summary, we have shown that the fusion of IL-2 to an intact Ab molecule has changed its binding properties to FcR-bearing cells and that this is correlated with changes in biodistribution and pharmacokinetic properties *in vivo*. This effect can be reversed, in part, by using immunoglobulin C regions with naturally or artificially reduced affinity for FcR, in the construction of IL-2 based immunocytokines. In this report we describe results using C γ 4 and mutated C γ 1 H chains. We have also found that C γ 2-IL-2 immunocytokines, as well as mutants with further reduction in FcR binding (17), have the same extended half-lives in mice (data not shown). Along with these changes, the properties of longer circulating $t_{1/2}$ and enhanced uptake into tumor of these novel immunocytokines are correlated with im-

proved efficacy in treatment models where T-cells are likely to play a role.

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Cancer Res 1999;59:2159-2166.

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