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ORIGINAL ARTICLE

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Pharmacokinetics and stability of the ch14.18–interleukin-2 fusion protein in mice

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Abstract The fusion protein formed from ch14.18 and interleukin-2 (ch14.18–IL-2), shown to exhibit antitumor efficacy in mouse models, consists of IL-2 genetically linked to each heavy chain of the ch14.18 chimeric anti-GD2 monoclonal antibody. The purpose of this study was to determine the pharmacokinetics of ch14.18–IL-2 in mice and assess its stability in murine serum. Following i.v. injection, the fusion protein was found to have a terminal half-life of 4.1 h. Detection of IL-2 following injection of the ch14.18–IL-2 fusion protein showed a similar half-life, indicating that the fusion protein prolongs the circulatory half-life of IL-2. Detection of human IgG1 following injection of ch14.18–IL-2 showed a terminal half-life of 26.9 h. These data suggested that the native fusion protein is being altered in vivo, resulting in a somewhat rapid loss of detectable IL-2, despite prolonged circulation of its immunoglobulin components. In vitro incubation of the ch14.18–IL-2 fusion protein in pooled mouse serum at 37 °C for 48 h resulted in a loss of its IL-2 component, as detected in enzyme-linked immunosorbent assay systems and in proliferation assays. Polyacrylamide gel electrophoresis and Western blot analysis of the fusion protein incubated in mouse serum at 37 °C indicated that the ch14.18–IL-2 is cleaved, resulting in a loss of the 67-kDa band (representing the IL-2 linked to the IgG1 heavy chain) and the detection of a band of more than 50 kDa, slightly heavier than the IgG1 heavy chain itself. This suggests that the fusion protein is being cleaved in vitro within the IL-2 portion of the molecule. These studies show that (1) ch14.18–IL-2 prolongs the circulatory half-life of IL-2 (compared to that of soluble IL-2) and (2) the in vivo clearance of the fusion protein occurs more rapidly than the clearance of the ch14.18 antibody itself, possibly reflecting in vivo cleavage within the IL-2 portion of the molecule, resulting in loss of IL-2 activity.

Key words Interleukin-2 · Antitumor antibody · Targetted immunotherapy · Ganglioside.

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Introduction

Monoclonal antibodies to tumor-associated antigens have been studied as a treatment for human malignancies, either alone [15] or in combination with cytokines, such as interleukin-2 (IL-2) [1]. In an effort to improve on antibody-mediated antitumor activity, fusion proteins have been created in which a cytokine is directly linked to a tumor-reactive antibody [10, 24]. This report evaluates the pharmacokinetics of the ch14.18–IL-2 fusion protein, consisting of IL-2 linked to the ch14.18 chimeric antibody, which binds the GD2 disialoganglioside expressed on human neuroblastoma, melanoma, and certain other tumors [3, 26].

Ch14.18–IL-2 is a genetically engineered protein containing the ch14.18 antibody with one molecule of IL-2 at the carboxy terminus of each IgG1 heavy chain [10]. In vitro studies have shown binding of the antibody portion of the fusion protein to GD2-positive cells, with the linkage between the ch14.18 and IL-2 resulting in a marked increase in antibody binding as determined by direct and competitive binding assays [10, 13, 23].

Cultured $GD2^+$ tumor cells coated with the fusion protein were also killed by T cells and natural killer (NK) cells at a higher rate than uncoated cells [10, 13, 23]. The IL-2 component of the fusion protein is active at stimulating IL-2-responsive cells when used as the soluble or tumor-cell-bound ch14.18–IL-2 fusion protein [13]. In vivo studies have demonstrated that intravenous (i.v.) injection of this fusion protein can cause complete inhibition of tumor xenografts in severe combined immunodeficient (SCID) mice [3, 25]. In a syngeneic murine neuroblastoma model, the fusion protein could eliminate established liver and lung metastases and prolong survival [19]. A therapeutic advantage of the recombinant ch14.18–IL-2 fusion protein over a mixture of cytokine and antibody has been demonstrated in mouse models [19]. Thus, significant antitumor activity has been demonstrated both in vitro and in vivo, with the fusion protein exhibiting a greater degree of in vivo tumor destruction than the simultaneous administration of equal amounts of ch14.18 antibody and soluble IL-2.

As antitumor responses have been demonstrated in murine models, understanding the mechanisms of this response should help in the extrapolation of these treatments to human clinical trials. In vivo analyses in nude, SCID, and T-cell- or NK-depleted mice have shown that NK cells, rather than T cells, are essential in the activity of the ch14.18–IL-2 protein against a syngeneic $GD2^+$ murine neuroblastoma [20]. The in vivo pharmacokinetic pattern and clearance of the ch14.18–IL-2 fusion protein affect the dosing intervals necessary to attain and maintain a therapeutic level of the drug. IL-2 is known to be cleared rapidly ($t_{1/2} \cong 1$ h) [6, 17, 22], while immunoglobulins are cleared more slowly ($t_{1/2}$ of 1 day or greater) after i.v. bolus injections. It was important to determine which, if either, of these patterns would approximate to the in vivo clearance of the intact fusion protein. We have developed a sensitive, specific and quantitative method of analyzing both the native form of the fusion protein and its IL-2 and antibody components [9]. This system of analysis has allowed us to evaluate the clearance of the fusion protein and its components in a murine model system where reproducible antitumor responses have been demonstrated. The pharmacokinetics of the IL-2 and ch14.18 components of the ch14.18–IL-2 fusion protein were also analyzed and compared to the pharmacokinetics of both the native ch14.18 antibody and soluble IL-2. These studies demonstrated that the clearance of the intact ch14.18–IL-2 fusion protein is far more rapid than that of the immunoglobulin itself, and results, at least in part, from the gradual loss of detectable IL-2 from the fusion protein circulating in mice. In vitro studies were performed to assess the potential role of mouse serum in the metabolism and clearance of the ch14.18–IL-2 fusion protein.

Materials and methods

Drug formulation and production

Ch14.18–IL-2

The ch14.18–IL-2 fusion protein has been described previously [10]. Its purification was performed at the NCI Monoclonal Antibody and Recombinant Protein (MARP) production facility (Frederick, Md.). The stock concentration was 0.4 mg/ml. The drug was stored frozen at −80 °C until use. An aliquot of the thawed protein was analyzed by polyacrylamide gel electrophoresis (PAGE) and Western blot prior to injection into the mice, to determine the purity [9]. A humanized form of this same fusion protein was made by CDR grafting of the antigen-binding sites of the murine hypervariable heavy and light chain regions from the 14.18 murine mAb into these locations on a human IgG1 immunoglobulin, also containing an IL-2 molecule (human) at the carboxy terminus of each IgG heavy chain (S. Gillies, unpublished results). This molecule was produced in NS/O cells, purified at the NCI (Frederick, Md.), and designated as hu14.18–IL-2.

IL-2

Human recombinant IL-2 (Hoffmann La Roche, Nutley, N.J., obtained via the NCI) was used. This was reconstituted with phosphate-buffered saline (PBS) at a concentration of 600 000 IU/100 µl and stored at 4 °C until use.

Ch14.18

The ch14.18 antibody, produced by Repligen (Needham, Mass.) for the NCI was stored at −80 °C at a concentration of 5.2 mg/ml and thawed prior to use. Purity of the sample was evaluated by PAGE and Western blot analysis. When necessary, the samples were further diluted with PBS prior to injection into the mice.

Animals

All in vivo studies used female Balb/C mice, 6–8 weeks of age (Harlan Sprague Dawley). The mice received either 20 µg ch14.18–IL-2, 16 µg ch14.18, or 3 µg IL-2 through i.v. injection in 50-µl volumes from a 30-gauge needle. Ether was used for anesthesia. The animals were given free access to food and water. Approval of the University of Wisconsin animal-use committee was obtained.

In vivo dosing and sample collection

Equivalent concentrations of ch14.18–IL-2 or ch14.18 or IL-2 were calculated on the basis of ch14.18 representing 5/6 of the molecular mass of the ch14.18–IL-2 fusion protein and IL-2 representing 1/6 the molecular mass of the intact fusion protein. At specified times (ranging from 0 to 144 h) blood was obtained via retro-orbital collection. Repetitive blood draws were performed on five mice per treatment group for each assay time. Each blood sample was stored on ice (for up to 15 min), until all samples were collected, and then centrifuged (12 000 *g*, 5 min, 4 °C). Serum was separated from the clotted blood and stored at −20 °C until assayed.

Drug level monitoring

The concentrations of human IgG1, IL-2 and intact ch14.18–IL-2 fusion protein in the serum specimens were evaluated using the following ELISA methods developed for this purpose [9].

1A7/IL-2 enzyme-linked immunosorbent assay (ELISA)

1A7/IL-2 ELISA for the measurement of the intact ch14.18–IL-2 fusion protein was described previously [9]. The capture antibody used was 1A7, a mouse monoclonal anti-idiotype to 14.G2a antibody [27]. The secondary reagent used was a goat anti-(human IL-2) antibody coupled to biotin (R & D Systems, Minneapolis, Minn.). The assay was calibrated using serial dilutions of ch14.18–IL-2 fusion protein (MARP lot 1).

IgG1 ELISA

IgG1 ELISA for detection of the hIgG-1 component of the fusion protein was performed as previously described [9], using a goat anti-(human IgG) antibody (Southern Biotech Associates, Birmingham, Ala.) as a capture antibody, and a sheep anti-(human IgG1) antibody coupled to horseradish peroxidase (The Binding Site Ltd., Birmingham, England) as a secondary reagent. As a standard in this assay, ch14.18 from a purified stock of 5.34 mg/ml was used.

IL-2 ELISA (detects total human IL-2)

The third detection system used was the commercially available human IL-2 ELISA (Immunotech, France). The assay procedure was performed according to the manufacturer's manual. This kit was calibrated against the WHO International Standard for IL-2, and contained microtiter plates coated with a mouse monoclonal anti-(human IL-2) antibody. A different monoclonal anti-(human IL-2) linked to acetylcholinesterase was used as the reporter antibody. After washing, bound enzymatic activity was measured after the addition of a chromogenic substrate provided in the kit.

Pharmacokinetic data analysis

The drug delivery and sample collection intervals were closely monitored. Assay times were calculated as the time from injection to collection. Half-life calculations were performed using PKAnalyst. The plots of serum concentration against time after i.v. bolus administration were fitted by non-linear regression to either a two- or three-compartment model based on the best fit. The half-lives and area under the curve (AUC) were calculated as previously defined [5]. The concentration of IL-2, IgG1, and intact fusion protein at time zero was extrapolated on the basis of the calculated $t_{1/2}$ at the first measurable assay point.

Serum preparation

Pooled mouse serum (Sigma, St. Louis, Mo.) was used for in vitro experiments. For some studies, where indicated, aliquots were heat-inactivated at 56 °C for 30 min or 65 °C for 1 h with inversion of the tubes every 15 min to mix the serum. Following the heat inactivation, the serum was stored at 4 °C until used in the stability assays.

In vitro stability

Ch14.18–IL-2 at a concentration of 23 µg/ml was added to either normal or heat-inactivated mouse serum at 4 °C, prepared as stated above. The serum mixtures were incubated at either 4 °C, 25 °C or 37 °C. Samples were collected after 0, 4, 8, 16, and 24 h, immediately frozen on solid CO_2, and stored at −70 °C until analyzed in the 1A7/IL-2 ELISA. In separate experiments, fusion proteins (ch14.18–IL-2 or hu14.18–IL-2) were incubated in normal pooled mouse serum (2 µg fusion protein/300 µl serum). Samples were incubated at either 4 °C or 37 °C for 0, 24, or 48 h, removed, immediately frozen on solid CO_2, and stored at −70 °C until use. Samples were evaluated in the 1A7/IL-2 and IL-2 ELISA systems.

Proliferation assays

A 72-h proliferation assay was used to investigate the biological activity of the ch14.18–IL-2 or hu14.18–IL-2 fusion proteins after incubation in mouse serum [13]. Tf1-β, a myeloid leukemic cell line expressing intermediate-affinity IL-2 receptor complexes, was used as the responding cell [7, 8]. The hu- or ch14.18–IL-2 fusion protein or IL-2 alone was first incubated in 100% pooled mouse serum (Sigma, St. Louis, Mo.) at a concentration of 1×10^5 units of IL-2/ml for 24 h at either 4 °C or 37 °C. These specimens were then diluted in tissue-culture medium and tested for their ability to induce proliferation.

Flow-cytometric detection of fusion protein

The ability of the fusion protein to bind to GD2-positive tumor cells and to deliver IL-2 to the tumor cell surface was assessed by flow-cytometric analysis as published previously [13]. The ch14.18–IL-2 or hu14.18–IL-2 fusion protein (33 µg/ml) was incubated in 100% mouse serum or standard tissue-culture medium for 24 h at either 4 °C or 37 °C. Either ch14.18–IL-2 or hu14.18–IL-2 without incubation in mouse serum was used as a control. Aliquots of fusion protein (1.5 µg) were incubated with 5×10^5 M-21 human melanoma cells for 30 min at 4 °C. Binding of the fusion protein to the cells was measured by indirect staining, detecting the IgG components with fluorescein-conjugated goat anti-(human IgG) (Caltag, San Francisco, Calif.). The IL-2 component of the fusion protein was detected either by a polyclonal rabbit anti-(human IL-2) antibody (Endogen, Woburn, Mass.) followed by a phycoerythrin(PE)-conjugated goat anti-(rabbit Ig) antibody (Southern Biotechnology Associates Inc., Birmingham, Ala.), or by a monoclonal rat anti-(human IL-2) PE-conjugated antibody (PharMingen, San Diego, Calif.).

Sodium dodecyl sulfate/PAGE (SDS-PAGE)

The purity and structural integrity of the ch14.18–IL-2 fusion protein were evaluated by discontinuous PAGE under denaturing conditions [9, 18].

Western blot

For specific evaluation of the fusion protein components, a semi-dry blotting system was used as described previously [9, 29]. To immunoprobe the IgG1 component of the fusion protein, a sheep anti-(human IgG1) antibody coupled to horseradish peroxidase (The Binding Site, Birmingham, England), diluted at 2 µg/ml in TRIS/NaCl/0.05% Tween-20 was used, followed by Super Signal chemiluminescent substrate (Pierce, Rockford, Ill.) [9].

Results

Pharmacokinetics of the ch14.18–IL-2 fusion protein

The in vivo metabolism and clearance of the ch14.18–IL-2 fusion protein after bolus i.v. injection was compared to that seen with bolus i.v. injections of IL-2 or of ch14.18. The injection was well tolerated in the mice with no observable indications of toxicity. The ch14.18–IL-2 levels in animals receiving i.v. fusion protein were evaluated with an assay requiring detection of both IL-2 and ch14.18 idiotypic determinants on the same molecule (the 1A7/IL-2 ELISA). IL-2 levels in animals receiving IL-2 were measured in the IL-2 ELISA . Ch14.18 levels in animals receiving ch14.18 were measured in the

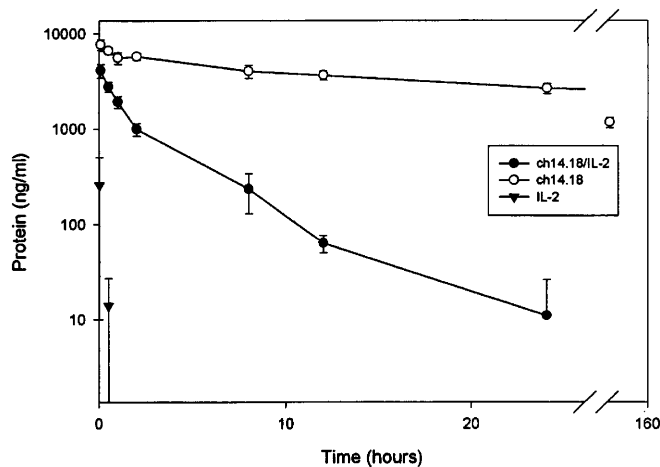

Fig. 1 Serum levels of interleukin-2 (*IL-2*), ch14.18, and ch14.18–IL-2 following i.v. injection of each. Separate groups of mice were injected in the tail vein with 20 μg ch14.18–IL-2, 16 μg ch14.18, or 3 μg IL-2 such that equivalent doses of immunoglobulin or cytokine were received. There were five mice per treatment group for each assay time. Ch14.18–IL-2 levels were assayed by the 1A7/IL-2 enzyme-linked immunoassay (ELISA), ch14.18 levels were assayed by the IgG1 ELISA, and IL-2 levels were measured by the IL-2 ELISA. Note that standard deviation (SD) bars are shown for all points, but are obscured by the symbols when SD values are small. Note also that the 144-h sample for the ch14.18–IL-2 value, and the 2-h and 12-h values for IL-2 are below the detection limits of the assay, and are not shown. *Bars* mean ± SD

IgG1 ELISA system. As shown in Fig. 1, the ch14.18–IL-2 fusion protein was cleared from the serum more rapidly than was the ch14.18 antibody and more slowly than free IL-2. The mean serum levels for the intact fusion protein followed a biphasic clearance pattern through 24 h with a terminal half-life of 4.1 h. The ch14.18 antibody exhibited a slower clearance, with a terminal half-life of 118 h. While the immunoglobulin was still detectable at the 144-hour assay time, the serum level of the intact ch14.18–IL-2 fusion protein, as detected in the 1A7/IL-2 ELISA was below detectable limits after 144 h. IL-2 was cleared from the serum rapidly and was below detectable limits within 2 h of injection. The area under the curve (AUC) is much lower for the ch14.18–IL-2 fusion protein (7437 ng ml^{-1} h^{-1}) than for the ch14.18 immunoglobulin (499 688 ng ml^{-1} h^{-1}). The ch14.18 immunoglobulin was still detectable after 12 days, thus the AUC for ch14.18 was extrapolated.

Alteration of the ch14.18–IL-2 fusion protein in vivo

The rapid loss of detectable intact ch14.18–IL-2 fusion protein, compared to the loss of detectable ch14.18 suggested that the fusion protein was being eliminated more rapidly than the free immunoglobulin ch14.18. Alternatively, the fusion protein was being modified (catabolized) in vivo, such that the intact IgG1 and IL-2 components were not being detected simultaneously on the same molecule. To distinguish these possibilities, the in vivo stability of the ch14.18–IL-2 fusion protein was assessed by measuring the concentration of the intact fusion protein and its components in mouse serum at various times after administration of a single bolus i.v. dose of 20 μg ch14.18–IL-2 (Fig. 2A). While the 1A7/IL-2 ELISA quantifies the intact native fusion protein, analyses of the serum from mice treated with ch14.18–

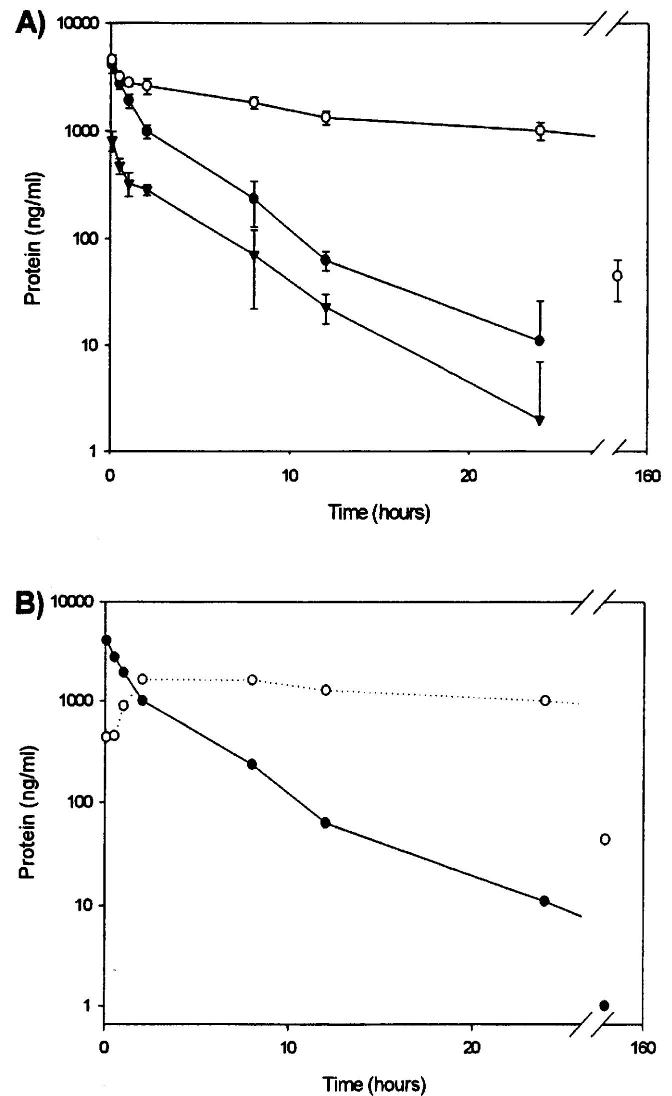

Fig. 2 A ELISA detection of IL-2, IgG1, and intact fusion protein following i.v. injection of ch14.18–IL-2. Samples from the Balb/c mice injected with 20 μg ch14.18–IL-2, described in Fig. 1, were also assayed in the IgG1 ELISA or IL-2 ELISA. The 1A7/IL-2 ELISA data are repeated from Fig. 1. Serum values for the IL-2 and 1A7/IL-2 assays were below detection limits for samples obtained at 144 h, and are not shown. **B** Serum level of IgG1 not detected by fusion protein assay. Data from Fig. 1 are replotted to show the IgG1 component not detected by the 1A7/IL-2 assay. (Total immunoglobulin detected in the IgG1 assay minus fusion protein immunoglobulin detected in the 1A7/IL-2 assay = immunoglobulin not detected by 1A7/IL-2 ELISA.) • Immunoglobulin detectable by 1A7/IL-2 ELISA, ○ immunoglobulin not detected by 1A7/IL-2 ELISA

IL-2 in the IgG ELISA and IL-2 ELISA might detect the presence of the IgG and IL-2 catabolites of the fusion protein not detected by the 1A7/IL-2 ELISA if the intact fusion protein were modified such that it ceased to be recognized by either the IL-2 or anti ch14.18 idiotype antibodies.

Figure 2A demonstrates that the concentration of native fusion protein (also shown in Fig. 1) decreases over time with a terminal half-life of 4.1 h. Note that the IgG1 assay should detect the amount of ch14.18–IL-2 that is detectable with the 1A7/IL-2 ELISA, as well as the ch14.18 components of the fusion protein which might no longer be detectable in the 1A7/IL-2 assay if there were any alteration in the IL-2 component of the molecule. Similarly, the IL-2 assay should detect the IL-2 as part of the fusion protein (detectable in the 1A7/IL-2 assay), and might also detect any soluble IL-2 potentially released from the fusion protein. Thus, both the IL-2 and IgG assays should detect the intact fusion protein. If the intact fusion protein is cleared from the serum as an intact protein (rather than being catabolized), the clearance curves for the ch14.18–IL-2 fusion protein should be identical in all three ELISA assays (1A7/IL-2, IgG, and IL-2 ELISA assays). The results shown in Fig. 2A demonstrate that identical curves were not observed.

The IL-2 assay shown in Fig. 2A demonstrates that IL-2, as a component of the fusion protein, has a half-life of 3.1 h, which is longer than previously demonstrated with free IL-2 following an i.v. injection measured by the same assay (shown in Fig. 1). This is similar to the half-life of the intact fusion protein as measured by the 1A7/IL-2 assay, and suggests that the IL-2 assay (Fig. 2A) is primarily detecting IL-2 as a component of the intact fusion protein in the serum of these treated animals. The IL-2 assay was standardized and calibrated with soluble IL-2. The values shown in the assay in Fig. 2A are therefore plotted as ng/ml IL-2. However, the IL-2 component of the fusion protein reflects only 1/6 of its molecular mass. If the mean serum values of IL-2 shown in Fig. 2A were actually multiplied by six to reflect the level of fusion protein detected by this IL-2 assay, the clearance profile of the ch14.18–IL-2 fusion protein detected by the 1A7/IL-2 ELISA would be nearly superimposable with the curve obtained for this same fusion protein as measured by the IL-2 ELISA. This result suggests that free IL-2 (which should be cleared rapidly as shown in Fig. 1) does not remain in the serum as a molecule distinct from the intact fusion protein, following i.v. injection of fusion protein.

In contrast, human immunoglobulin, as detected by the IgG1 ELISA in these animals receiving an i.v. bolus of ch14.18–IL-2 fusion protein, demonstrates a markedly prolonged terminal half-life of 26.9 h, and was detected long after the intact fusion protein and IL-2 were no longer detectable. This indicates that a component of the ch14.18–IL-2 fusion protein, which is not detected by the IL-2 or 1A7/IL-2 ELISA, remains in the circulation longer.

To define better the clearance of the metabolic products of the ch14.18–IL-2 fusion protein, the level of immunoglobulin detected as a component of intact fusion protein (from the 1A7/IL-2 assay) was plotted separately and these values were subtracted from the total IgG1 detected (by the IgG1 assay), thus quantifying the serum level of ch14.18 undetectable in the intact fusion protein assay (Fig. 2B). These results confirm that the ch14.18–IL-2 fusion protein is altered in vivo. As demonstrated by the early decrease in the ch14.18–IL-2 level, there is an early increase in the concentration of the immunoglobulin component detectable only in the IgG1 ELISA. Clearance of this component of the ch14.18–IL-2 fusion protein, detected by IgG ELISA (but not 1A7/IL-2 ELISA), was simultaneously analyzed and it was found to have a terminal half-life of 26.9 h, somewhat shorter than that seen with the native ch14.18 antibody (terminal half-life 118.5 h in Fig. 1). These results suggest that the intact fusion protein is altered in vivo, causing it to lose its detectibility by IL-2 ELISA, and by 1A7/IL-2 ELISA, and resulting in a half-life of 3–4 h.

In vitro detection of the ch14.18–IL-2 fusion protein

The results of the in vivo pharmacokinetics shown in Fig. 2A, B suggest the fusion protein is modified in vivo. To evaluate some of the factors potentially responsible for the in vivo modification of the fusion protein, its stability was assessed in vitro in mouse serum (Fig. 3). When added to normal mouse serum, the intact fusion protein remains stable at 4 °C and 25 °C through the 48 h tested. When the incubation was in normal mouse serum at 37 °C, the ability to detect intact fusion protein with the 1A7/IL-2 ELISA system decreased over time, but more slowly than the decrease seen in vivo

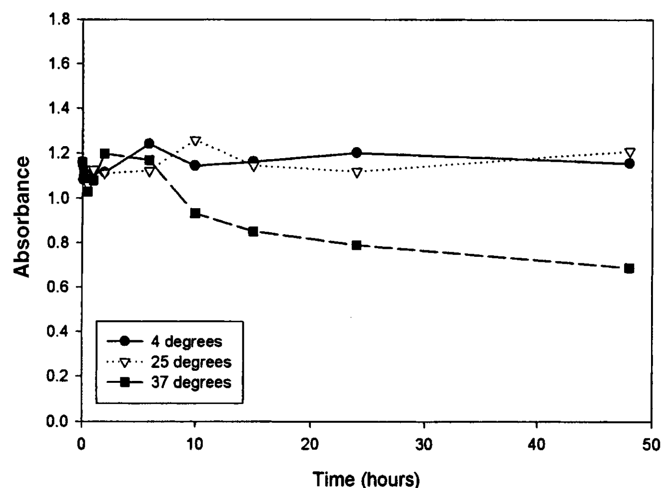

Fig. 3 Temperature-dependent stability of ch14.18–IL-2 fusion protein in normal mouse serum. Ch14.18–IL-2 (23 μg/ml) was added to normal mouse serum. These samples were then incubated at 4 °C, 25 °C, or 37 °C. Samples were collected at 0, 0.08, 0.5, 1, 2, 6, 10, 15, 24, and 48 h, frozen, and analyzed in duplicate in the 1A7/IL-2 ELISA. Data are representative of two experiments

(Fig. 2A). A difference in intact fusion protein levels can be seen from 8 h through 24 h of in vitro incubation in serum. As this loss of detection of the ch14.18–IL-2 molecule occurs in mouse serum at 37 °C but not 25 °C, mouse serum was first heat-inactivated to inhibit temperature-sensitive enzymatic reactions. As noted in Fig. 4A, B the loss of detectable ch14.18–IL-2 could not be abolished by heat inactivation of the mouse serum at 56 °C or 65 °C.

In vitro detection of the IL-2 component of the ch14.18–IL-2 fusion protein

The in vivo data shown in Fig. 2A, B suggest that the structural change in the fusion protein occurring in the mouse is a result of changes within the IL-2 portion. To evaluate the potential role of mouse serum in this alteration, either ch14.18–IL-2 or hu14.18–IL-2 was added to normal pooled mouse serum and incubated at 4 °C, 25 °C or 37 °C for 0, 24 or 48 h. The fusion proteins remained detectable at 4 °C (Fig. 5) and 25 °C (not shown) throughout the 48 h tested. When the fusion protein was incubated at 37 °C, the concentration of IL-2 decreased in the first 48 h, following a pattern similar to that measured in the 1A7/IL-2 ELISA. This confirms the temperature-dependent loss in mouse serum of antigenic sites on the human IL-2 component of the ch14.18–IL-2 and hu14.18–IL-2 fusion proteins, recognizable by either the monoclonal or polyclonal anti-(human IL-2) antibodies used in these ELISA systems.

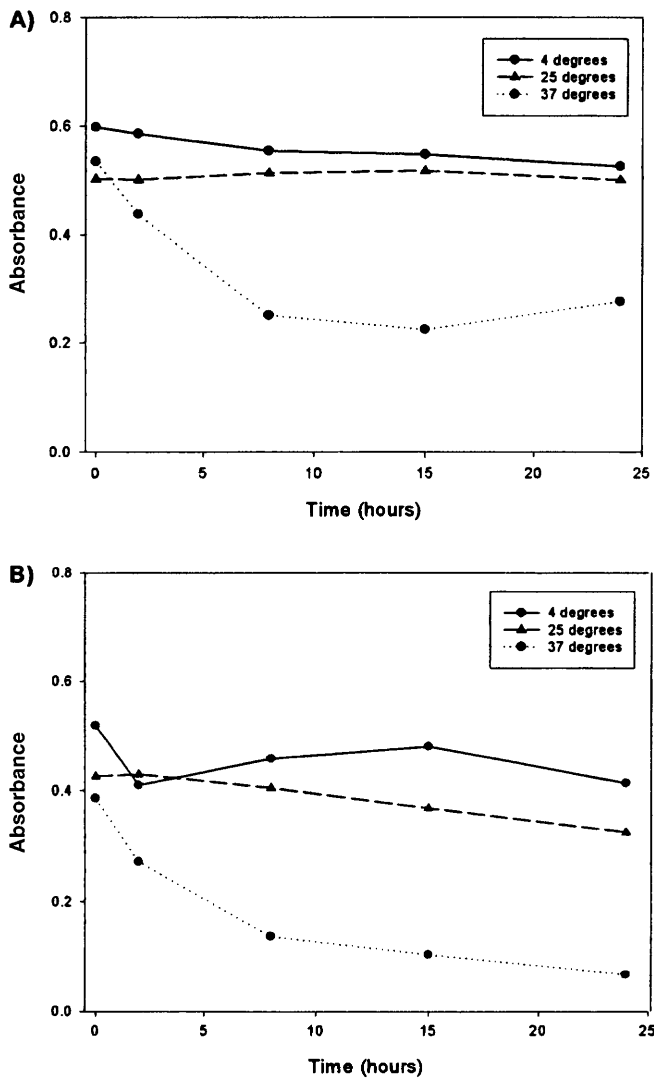

Fig. 4A, B Temperature-dependent stability of ch14.18–IL-2 fusion protein in heat-inactivated mouse serum. Ch14.18–IL-2 (23 μg/ml) was added to mouse serum heat-inactivated at (**A**) 56 °C for 30 min or (**B**) 65 °C for 1 h. These samples were then incubated at 4 °C, 25 °C or 37 °C. Samples were collected at 0, 2, 8, 15 and 24 h, frozen and analyzed in duplicate in the 1A7/IL-2 ELISA. Data are representative of two experiments

In vitro inactivation of IL-2 activity

The effects of preincubation in mouse serum on the function of both IL-2 and the hu14.18–IL-2 fusion protein were tested in a 72-h proliferation assay. Figure 6A shows the results with IL-2 after prior incubation in medium or mouse serum at 4 °C or 37 °C. The results with the ch14.18–IL-2 fusion protein are pre-

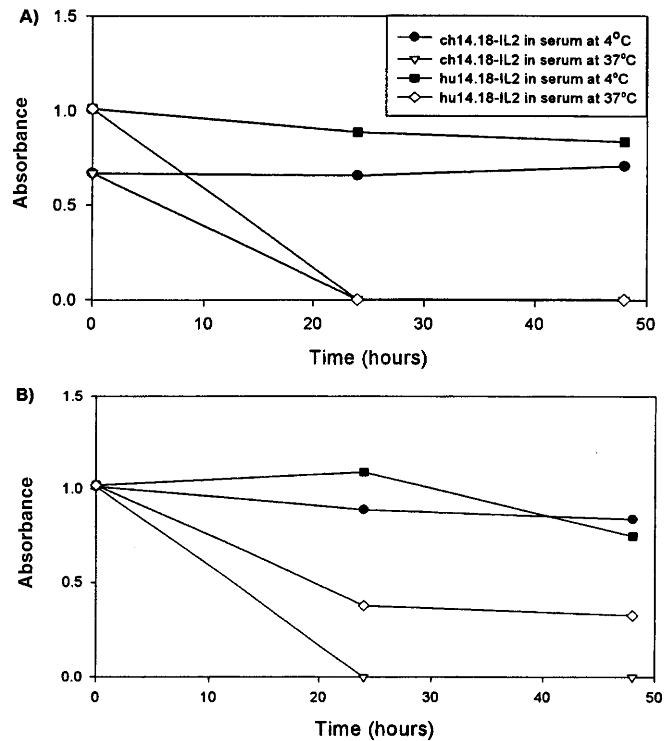

Fig. 5A, B Comparison of fusion protein detected in 1A7/IL-2 and IL-2 ELISA after incubation in normal mouse serum. Either ch14.18–IL-2 or hu14.18–IL-2 was added to normal mouse serum to a concentration of 23 μg/ml. These samples were then incubated at 4 °C or 37 °C. Samples were collected at 0, 24 or 48 h, frozen, and analyzed in duplicate in either the 1A7/IL-2 ELISA (**A**) or the IL-2 ELISA (**B**). Ch14.18–IL-2 in serum at 4 °C, ch14.18–IL-2 in serum at 37 °C, hu14.18–IL-2 in serum at 4 °C, hu14.18–IL-2 in serum at 37 °C. Data are representative of two experiments

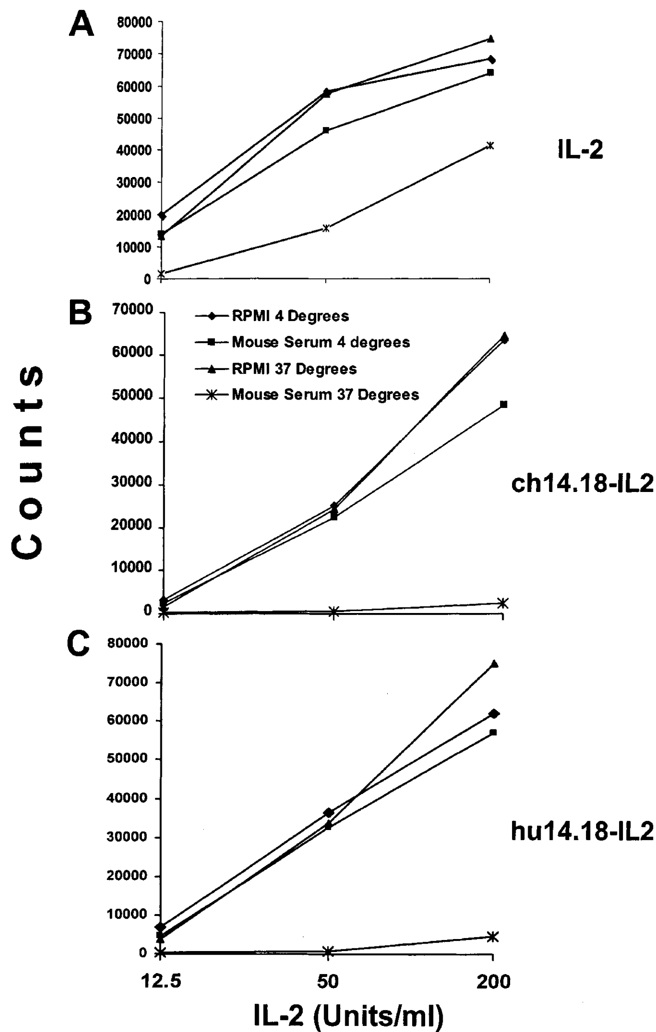

Fig. 6A–C Comparison of functional activity of (**A**) IL-2, (**B**) ch14.18–IL-2, (**C**) hu14.18–IL-2 after incubation in mouse serum. Either IL-2 or fusion protein (1×10^5 units IL-2/ml) was incubated in 100% mouse serum or RPMI medium containing 10% human serum at 4 °C or 37 °C for 24 h. These specimens were then diluted and used to stimulate IL-2 -responsive Tf-1β cells in vitro in a 72-h proliferation assay. Data are representative of four experiments

sented in Fig. 6B, and those with hu14.18–IL-2 in Fig. 6C. These results show that incubation of the chimeric or humanized form of the fusion protein in mouse serum, at 37 °C for 24 h, is associated with nearly complete loss of functional IL-2 activity. In contrast, IL-2 itself shows only partial loss of activity after 24 h at 37 °C in mouse serum, detected primarily at the lower IL-2 concentrations.

In separate experiments, ch14.18–IL-2 was incubated at 37 °C for 24 h in various concentrations of mouse serum, ranging from 100% to 10%. Mouse serum exhibited an inhibitory effect on the functional activity of the ch14.18–IL-2 fusion protein, as measured by proliferation assays, in a concentration-dependent manner (data not shown). The ability to detect the ch14.18–IL-2 by IL-2 ELISA was similarily inhibited at the higher concentrations of mouse serum in a dose-dependent manner. When the mouse serum was diluted below 10%, the 37 °C 24-h incubation no longer inhibited detection of ch14.18–IL-2 by ELISA (data not shown).

Incubation of the fusion protein in 100% mouse serum at 37 °C resulted in both a loss of native fusion protein, as detected in the 1A7/IL-2 ELISA, and a loss of functional IL-2 activity in proliferation assays. Flow-cytometric evaluation was also used to assess the ability of the fusion protein (ch14.18–IL-2 and hu14.18–IL-2) to bind to GD2-positive cells after incubation in 100% mouse serum. Figure 7 demonstrates that the fusion protein binding to the GD2-positive cells can be detected by recognition of its immunoglobulin component (Fig. 7A), and by its IL-2 component (Fig. 7B, C). The binding of the functional protein to the tumor cells is not altered by prior incubation of the fusion protein in mouse serum at 4 °C or 37 °C (Fig. 7A). In contrast, the ability to detect IL-2 bound to the tumor cells is dramatically inhibited following incubation at 37 °C but not at 4 °C. This is demonstrated directly with conjugated monoclonal antibody (Fig. 7B) and indirectly with polyclonal antibody against IL-2 (Fig. 7C). The mouse serum appears to alter the hu14.18–IL-2 and the ch14.18–IL-2 in a similar manner. This result indicates that the 24-h 37 °C incubation in mouse serum does not interfere with the ability of the fusion proteins to bind to $GD2^+$ tumor cells, but it does cause a decrease in detectible IL-2 delivered to the tumor cells by the fusion proteins.

SDS-PAGE and Western blot analysis

The components of the ch14.18–IL-2 fusion protein were detected by PAGE and further assessed with Western blot analysis (Fig. 8). Ch14.18–IL-2 was evaluated by electrophoresis under reducing conditions, using molecular mass markers as standards. The protein bands located at 67 kDa represent the heavy chains of ch14.18 antibody attached to IL-2 (Fig. 8A, lane 1). Recognition of the heavy chains by horseradish-peroxidase-conjugated anti-(human IgG1) indicates that the 67-kDa bands noted in the ch14.18–IL-2 samples contain human immunoglobulin (Fig. 8A, lanes 1, 2, 3, and fainter bands in lanes 3 and 4). These correspond to the IgG heavy chain linked to IL-2. Incubation of the ch14.18–IL-2 in mouse serum at 37 °C for 48 h resulted in a decrease in the intensity of the 67-kDa band, with a concominant formation of a new band measuring slightly more than 50 kDa (Fig. 8A, lane 4) Addition of aprotinin prior to incubation in mouse serum prevented the development of this approximately 55-kDa band (Fig. 8A, lane 5). This band seen in lane 4 has a slightly greater molecular mass than the heavy chain of the ch14.18 molecule (Fig. 8B, lanes 1, 2) itself. This result suggests that the band of about 55 kDa in lane 4 of Fig. 8A represents the heavy chain of the ch14.18–IL-2 molecule still containing a small (non-functional), residual component of an IL-2 molecule. These results

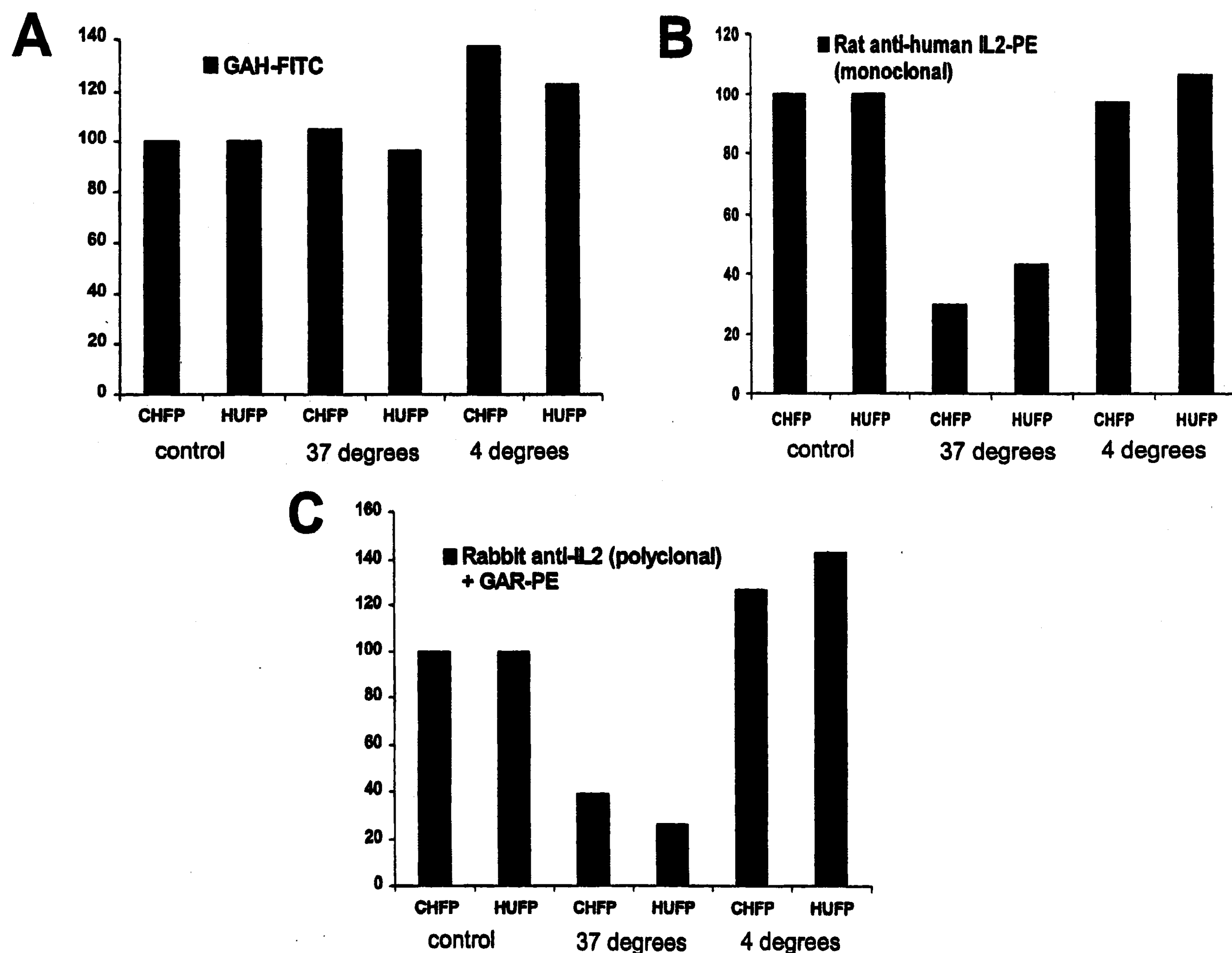

Fig. 7A–C Inhibition of IL-2 delivery to GD2-positive tumor cells by ch14.18–IL-2 (chFP) and hu14.18–IL-2 (huFP) after incubation in mouse serum. The fusion protein (FP) was incubated in 100% mouse serum for 24 h at 4 °C or 37 °C. Fusion protein mixed with 100% mouse serum without incubation was used as a control. Binding of the fusion protein to the M-21 cells was evaluated by flow cytometry using (**A**) fluorescein-isothiocynate-labelled goat anti-(human IgG1 (*GAH-FITC*), (**B**) monoclonal rat anti-(human IL-2) antibody conjugated to phycoerythrin (*PE*), and (**C**) rabbit anti-(human IL-2) antibody, followed by goat anti-(rabbit immunoglobulin) conjugated to PE (*GAR-PE*). Data are presented as a percentage of the control mean fluorescence intensity (*MFI*), where the control value, the MFI of the same fusion protein (chFP or huFP) mixed with mouse serum and not incubated, is defined as 100% (first two bars in each panel). Data represent the mean of four and five separate experiments for ch14.18–IL-2 and hu14.18–IL-2 respectively

are consistent with enzymatic cleavage occurring in mouse serum within the IL-2 portion of the molecule, which can be inhibited with aprotinin.

Discussion

Antitumor efficacy of the ch14.18–IL-2 fusion protein has been repeatedly demonstrated in mice [3, 25]. In vivo administration of this molecule is far more effective than equivalent amounts of ch14.18 plus soluble IL-2 against experimental and spontaneous metastases of human melanoma xenografts and syngeneic neuroblastoma [19, 20, 25]. A previously performed study reported the in vivo pharmacokinetics of the ch14.18–IL-2 fusion protein and free ch14.18 (detected by an ELISA for human IgG determinants [11]) and found the terminal half-lives were 20 h and 59 h respectively. These values are somewhat similar to the values of 26.9 h and 118 h obtained here by ELISA measurement of the IgG component of the ch14.18–IL-2 fusion protein injected into Balb/c mice. The calculated half-life of the fusion protein and ch14.18 antibody vary slightly with these different methods of analysis because of differences in the sensitivity and specificity of the detection systems.

The 1A7/IL-2 ELISA system used here enables the selective analysis of only the intact ch14.18–IL-2 fusion protein, which is recognised by both anti-IgG and anti-IL-2 antibodies [9] thus differentiating the intact fusion protein from that which is altered in vivo or in vitro. Following a single i.v. bolus injection, the terminal half-life of the ch14.18 chimeric antibody (118 h) was more

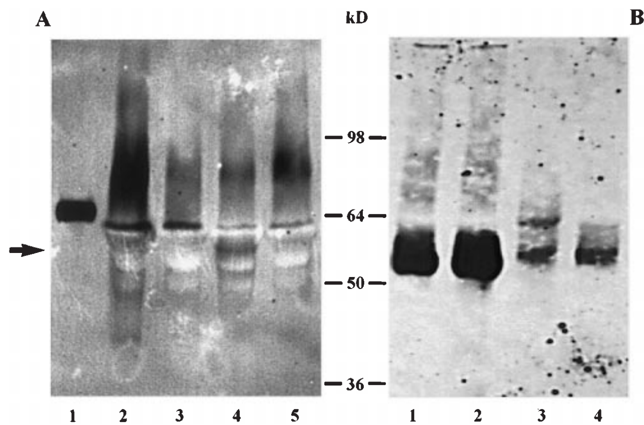

Fig. 8A, B Examination of the ch14.18–IL-2 fusion protein prior to (i.e. t_0) and after incubation (48 h) in mouse serum using sodium dodecyl sulfate/polyacrylamide gel electrophoresis followed by Western blot analysis. Anti-(human IgG1) antibody conjugated to horseradish peroxidase was used to identify the human immunoglobulin component of the fusion protein. Molecular masses were extrapolated using 98-kDa, 64-kDa, 50-kDa, and 36-kDa markers. **A** *Lane 1* ch14.18–IL-2 in TRIS buffer, *lane 2* ch14.18–IL-2 in mouse serum (t_0), *lane 3* ch14.18–IL-2 in mouse serum with aprotinin (t_0), *lane 4* ch14.18–IL-2 (after 48 h incubation in mouse serum at 37 °C), *lane 5* ch14.18–IL-2 (after 48 h incubation with aprotinin in mouse serum at 37 °C). The *filled arrow* on the *left* indicates the new 55- to 60-kDa band found in lane 4, the *empty arrow* indicates the position of the intact heavy chain of fusion protein with IL-2 attached. **B** *Lane 1* ch14.18 antibody in mouse serum (t_0), *lane 2* ch14.18 in mouse serum (after 48 h at 37 °C), *lane 3* fusion protein in mouse serum (t_0), *lane 4* fusion protein in mouse serum after 48 h at 37 °C

prolonged than that of the intact fusion protein (3–4 h). Clearance of the intact ch14.18–IL-2 molecule was more prolonged than that of free IL-2. As shown in mice and humans, IL-2 is rapidly cleared from the blood, with a $t_{1/2\alpha}$ ranging from 5 min to 30 min and a $t_{1/2\beta}$ ranging from 45 min to 120 min [16, 21, 22, 28]. The principal routes of IL-2 clearance from the serum are excretion through the kidney [6] and leakage from the intravascular compartment to the extravascular compartment [21]. On the basis of the radiolabelled IL-2 data, it was also hypothesized that the IL-2 was metabolized to constituent amino acids and recycled into new proteins [22]. The prolongation of the in vivo half-life of IL-2, when delivered i.v. as the ch14.18–IL-2 fusion protein, has also been demonstrated with other antibody/cytokine fusion proteins [4, 11, 12, 14]. In efforts to improve the therapeutic index of IL-2, to decrease toxicity and prolong exposure to IL-2, subcutaneous or intraperitoneal administration were used [2]. The prolonged terminal half-life of the fusion protein, relative to free IL-2, offers the potential benefit of prolonging the circulatory half-life of IL-2 following an i.v. bolus injection of fusion protein.

The ELISA data obtained with mouse serum specimens following i.v. injection, presented in this study, indicate that the ch14.18–IL-2 fusion protein is modified in vivo and suggests that this alteration occurs within the IL-2 portion of the protein. A somewhat analogous in vivo loss of detectable IL-2 fusion protein, but not IL-12 fusion protein has been noted in mice, with fusion proteins made with a separate antitumor antibody, KS1/4 [12]. These in vivo studies do not clarify the molecular mechanism for the in vivo modification of the fusion protein's IL-2 component, nor the in vivo location (i.e.: plasma, interstitial fluid, intracellular fluid) where the IL-2 component is modified. In order to evaluate reactions that might be occurring in the soluble component of the circulation, in vitro studies were performed in murine serum. Results show that the IL-2 component of this fusion protein is modified in mouse serum when incubated at 37 °C but far more slowly than is seen in vivo (compare Figs. 3, 4 with Fig. 2A). This suggests that a small component of the in vivo modification of the fusion protein detected here may be occurring directly in the vascular volume itself. Indeed, even soluble human IL-2 shows some instability in vitro when incubated at 37 °C in mouse serum (Fig. 5). Previous studies have demonstrated that the peptide bond in the ch14.18–IL-2 fusion protein, between the carboxyl terminus of the ch14.18 heavy chain and the IL-2, is susceptible to cleavage with plasmin [11]. An alternative explanation for the in vitro changes observed in the present study is that interaction with other proteins in the serum results in structural changes in IL-2 such that it is no longer detectable by our polyclonal or monoclonal antibodies used in the ELISA. This change in the IL-2 component of the fusion protein occurs while its immunoglobulin portion preserves the antigenic site necessary for recognition in our ELISA systems. While the ch14.18–IL-2 fusion protein maintains its native form when kept at 4 °C or at 25 °C in mouse serum, detection of the native intact fusion protein is lost over time at 37 °C. While this serum inactivation can be blocked with aprotinin, suggesting involvement of protease activity, this inhibitory activity is not abrogated by prior heat inactivation of the serum. An evaluation of the fusion protein incubated in mouse serum reinforces the in vivo data that suggest that there is an alteration in the IL-2 portion of the fusion protein. Following 24 h at 37 °C in mouse serum, the fusion protein shows a striking decrease in IL-2 detectible by ELISA, flow-cytometric and proliferative assays, while retaining its IgG component, and its ability to bind to $GD2^+$ cells (measured in ELISA and flow assays respectively). However, the strikingly slower loss of IL-2 in vitro (Figs. 3, 4) than in vivo (Fig. 2A), suggests that separate mechanisms are influencing the IL-2 component (S. Gillies, unpublished data).

The in vivo antitumor efficacy, documented for this ch14.18–IL-2 fusion protein in SCID xenografts [3, 24, 25] and in syngeneic murine models [19, 20], far exceeds the antitumor efficacy of equivalent amounts of soluble IL-2 and soluble ch14.18 antibody given concurrently but as separate molecules. This proves that the intact fusion protein is having a potent in vivo antitumor effect that requires the physical linkage of functional IL-2 to the anti-GD2 antibody. The observation that the IL-2

component of the fusion protein is apparently modified or inactivated in vivo with a half-life of 3–4 h indicates that the potent antitumor effect of the intact fusion protein likely involves its rapid delivery of IL-2 to the tumor sites, prior to the inactivation of the IL-2 component of the fusion protein. As clinical testing of this anti-GD2–IL-2 fusion protein will involve the application of a humanized form of this same chimeric fusion protein, analysis of the humanized form, particularly in primate pharmacokinetic studies, will be helpful in predicting the levels occurring in patients, and determining whether the inactivation of the IL-2 component of this fusion protein observed in vivo in mice, and in vitro in mouse serum, may also be found during clinical studies. Our preliminary results with the humanized 14.18–IL-2 fusion protein indicate that the temperature-dependent loss of detectible IL-2, seen after 24 h in mouse serum, is not observed when the incubation is in human serum (unpublished data). This suggests that the biologically active, complete fusion protein molecule remains longer in human serum than in mouse serum, and indicates the importance of further pharmacokinetic assessment in patients receiving the chimeric or humanized form of this fusion protein.

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