A Chimeric Lym-1/Interleukin 2 Fusion Protein for Increasing Tumor Vascular Permeability and Enhancing Antibody Uptake

Peisheng Hu, Jason L. Hornick, Michelle S. Glasky, Aoyun Yun, Mary N. Milkie, Leslie A. Khawli, Peter M. Anderson, and Alan L. Epstein

Department of Pathology, University of Southern California School of Medicine, Los Angeles, California 90033 [P. H., J. L. H., M. S. G., A. Y., M. N. M., L. A. K., A. L. E.], and Section of Pediatric Hematology/Oncology, Mayo Clinic, Rochester, Minnesota [P. M. A.]

ABSTRACT

A murine antihuman B-cell monoclonal antibody, Lym-1, has shown considerable promise for the treatment of human malignant lymphomas. To enhance its clinical potential, a genetically engineered fusion protein consisting of a chimeric Lym-1 (chl.lym-1) and interleukin 2 (IL-2) was tested for mediating cytotoxicity, increasing vasopermeability, and enhancing antibody uptake in human malignant lymphomas. The chl.lym-1/IL-2 fusion protein, which was expressed initially in a baculovirus system and more recently in the glutamine synthetase gene amplification system, was shown to be processed and assembled into a normal immunoglobulin monomer with two IL-2 molecules per antibody. It was found to be equivalent to the chl.lym-1 antibody in antigen-binding specificity and relative affinity. In addition, it maintains IL-2 cytokine activity as demonstrated by support of T-cell proliferation. Moreover, in antibody-dependent cellular cytotoxicity assays against Raji target cells, chl.lym-1/IL-2 had approximately 2-fold and 4-fold higher cytotoxicity than chl.lym-1 and murine Lym-1, respectively. Used as a pretreatment, chl.lym-1/IL-2 enhances the uptake of chl.lym-1 at the tumor site by altering the permeability of tumor vessels producing tumor-normal organ ratios of 420:1 for blood and 1708:1 for muscle at 3 days. The in vitro and in vivo activities of chl.lym-1/IL-2, therefore, suggest that this genetically engineered antibody fusion protein may represent a new immunotherapeutic reagent for the treatment of human malignant lymphomas.

INTRODUCTION

Several major obstacles have been identified that limit the amount of MAb3 that binds to tumor. These include antigenic heterogeneity, circulating free antigen, antigenic modulation, lack of tumor specificity, and low tumor uptake (1–4). Dosimetric calculations obtained from clinical studies in humans have shown that only ~0.01-0.1% of the injected antibody dose actually binds and accumulates in the tumor despite the use of high avidity MAbs to tumor antigens (5–8). It has long been known that two key parameters that control the uptake of macromolecules in tumors are blood flow and vascular permeability (9–12). On the basis of this information, we formulated the hypothesis that MAbs might be used as carriers of vasoactive and proinflammatory peptides to alter the blood flow and/or permeability of tumor vessels without affecting these parameters in normal tissues. This approach is unique in that it is aimed at altering the physiology of tumor vessels to enhance the tumor uptake of MAbs. Our previous studies indicated that one vasoactive agent, IL-2, is a highly potent inducer of vasopermeability and a promising reagent for this approach (13, 14). IL-2 is a potent biological mediator of the immune system, and it occupies a central role in the augmentation of cell-mediated immune responses. Its major functions include the proliferation of T lymphocytes (15) and the generation of nonspecific tumor killing by activated macrophages, LAK cells, and tumor-infiltrating lymphocytes (16). In addition to its cytokine activity, IL-2 has been shown to induce vascular permeability when administered systemically by causing the efflux of intravascular fluids to the extravascular spaces (capillary leak syndrome; Refs. 17–20). In a previous study (14), our laboratory used a chemically produced Lym-1/IL-2 vasoconjugate as a pretreatment to increase uptake of a radiolabeled MAb tracer by 3–4-fold with no concomitant increase in uptake in normal organs. This was the first report of an immunoconjugate that specifically enhances tumor localization of blood-borne macromolecules. The mechanism of action of this immunoconjugate was attributed specifically to an increase in tumor vascular permeability.

These findings have important implications for the use of MAbs in the radioimmunodetection and therapy of cancer. In addition, IL-2 immunoconjugate pretreatment may potentially be used to improve the delivery of other biologically important molecules to tumor sites. The Lym-1/IL-2 immunoconjugate generated by chemical coupling methods was found, however, to be devoid of IL-2 cytokine activity with respect to T-cell proliferation and LAK cell generation. Thus, it appears that LAK cell generation is not a prerequisite for the vascular permeability changes. From the outset, it was believed that a completely functional IL-2 moiety would produce optimal vasopermeability effects at the tumor site compared to a chemically conjugated moiety. It was with these factors in mind that a fusion protein was constructed with chLym-1 and IL-2 to generate a vasopermeability-and cytokine-active chLym-1/IL-2, which can be used for treatment of the human malignant lymphomas.

MATERIALS AND METHODS

Reagents

Iodine-125 was obtained as sodium iodide in 0.1N sodium hydroxide, and chromium-51 was obtained as Na251CrO4 in normal saline (pH 8–10) from DuPont New England Nuclear (North Billerica, MA). Centricon concentrators were purchased from Amicon (Danvers, MA). Sephadex, buffer salts, and other reagents such as chloramine T, sodium metabisulfite, hydrogen peroxide, and 2,2'-azino-bis(3-ethylbenzthiazolinesulfonic acid) were purchased from Sigma Chemical Co. (St. Louis, MO).

The transfer vectors pBacPAK1 and pAcUW31, Bsa36 I-digested BacPAK6 viral DNA, and wild-type Autographa californica nuclear polyhedrosis virus (AcNPV, E2 strain) were obtained from Clontech Laboratories (Palo Alto, CA). Grace’s insect cell culture medium, gentamicin, and fungizone were purchased from Life Technologies (Gaithersburg, MD). Low melting gel agarose was obtained from FMC Bioproducts (Rockland, ME). Restriction endonucleases, T4 ligase, and other molecular biology reagents were purchased from New England Biolabs (Beverly, MA) or Boehringer Mannheim (Indianapolis, IN). Both BALB/c and athymic nude mice were purchased from Harlan-Sprague-Dawley (Indianapolis, IN).
Antibodies and Cell Lines

chLym-1 (IgG1κ) was constructed and expressed as described previously (21). MAb muLym-1 (IgG1κ) directed against a variant of the HLA-Dr antigen expressed in human B-cell lymphomas and muLym-2 directed against a different B-cell epitope (22) were obtained from Technicon International, Inc. (Tustin, CA). The murine B72.3 MAb directed against the TAG-72 antigen expressed on colorectal carcinoma cells (23) was obtained from CytoGen Corp. (Princeton, NJ), muLym-1 Fab', fragments and biotinylated muLym-1 were prepared as described previously (24, 25). muLym-1 anti-idiotypic MAb IA7 was produced and purified as described previously (21). 125I-labeled MAbs were prepared as described previously (21).

The Raji cell line, derived from an African Burkitt's lymphoma, was used to determine specific binding of Lym-1-derived antibodies (22). Raji cells were grown in RPMI 1640 (Irvine Scientific, Irvine, CA) supplemented with 10% fetal bovine serum (HyClone Laboratories, Logan, UT), l-glutamine, penicillin G (100 units/ml), and streptomycin (100 µg/ml).

SP9 (Clontech) and High Five (Invitrogen, San Diego, CA) cells were grown in EX-CELL 400 serum-free medium (JRH Biosciences) with fungizone (2.5 µg/liter) at room temperature in an orbital shaker. Viral stocks were produced by infecting attached cell cultures at a low multiplicity of infection and harvesting 2–4 days after infection.

Construction and Expression of chLym-1/IL-2 Fusion Protein

Construction of the expression vector was carried out using standard techniques. A BV transfer vector for chLym-1, pBVchLym-1 (21), was used as the parent vector. This vector carries the cDNA sequences for the human-mouse chLym-1 heavy chain under the control of the polyhedrin promoter and light chain under the control of the p10 promoter. Two oligonucleotides, 5'-GGAGAATCT-GGTGGGCGTGGCAAGCACCTTCTTCAAGTCA-3' and 5'-GTATCTACTGGTTCAAGTCAAGTGGGATGCT-3', were used to amplify the human IL-2 gene from a plasmid template obtained from the American Type Culture Collection (clone 67618; Rockville, MD). The PCR fragment was inserted into the SpeI site of pBVchLym-1, resulting in the BV transfer vector pBVchLym-1/IL-2, encoding the chimeric light chain, and a fusion protein consisting of the chLym-1 heavy chain with human IL-2 at its COOH terminus. Expression of chLym-1/IL-2 was carried out as described previously (21). Briefly, purified pBVchLym-1/IL-2 was cotransfected into SP9 cells with linear viral DNA BacPAK6 (Clontech) using Lipofectin (Life Technologies) according to the manufacturer's protocol. Five days after cotransfection, the virus-containing supernatant medium was collected, serially diluted, and used to infect fresh monolayers of SP9 cells. Three days after infection, 0.01% neutral red stain was added in the agarose overlay during plaque identification to assist in the visualization of recombinant virus. Several well-isolated plaques were selected, and small-scale infections with these putative recombinant viruses were performed to amplify the viruses. The recombinant BVs were identified by Western blot and ELISA for producing chimeric antibody.

Fusion Protein Purification

chLym-1/IL-2 was purified from the infected High Five cell culture medium by protein A affinity chromatography as described previously (21). Protein concentration of purified chLym-1/IL-2 preparations was determined spectrophotometrically. Purity of chLym-1/IL-2 was examined by SDS-PAGE of samples in reducing gels according to the method of Laemmli (26).

Immunosays

ELISA for chLym-1/IL-2. Identification of chLym-1/IL-2-containing supernatants was initially carried out by indirect ELISA with muLym-1 anti-idiotypic antibody IA7 essentially as described previously (21). Purified chLym-1 was used as the positive control, and wild-type BV supernatant was used as the negative control.

ELISA for IL-2. For the detection of the IL-2 portion of chLym-1/IL-2, plates were coated with MAb IA7, incubated with chLym-1/IL-2, chLym-1, or wild-type BV supernatant, as above. Rabbit anti-human IL-2 was used as the secondary antibody (BioSource International, Camarillo, CA), followed by horseradish peroxidase-conjugated goat anti-rabbit immunoglobulin. The antibody binding was developed and detected as described previously (21).

Raji Cell Competition RIA. The antigen-binding activity of chLym-1/IL-2 was determined by a competition RIA for binding to live Raji lymphoma cells. For these studies, 2 x 10^6 washed Raji cells were aliquoted to individual tubes and incubated with 20 ng of 125I-labeled muLym-1 and increasing serial dilutions of cold muLym-1, chLym-1/IL-2, or an irrelevant MAb (muLym-2). The cells and MAbs were incubated for 1 h at room temperature with constant mixing. After incubation, the cells were washed twice, and the cell pellet-associated radioactivity was measured in a gamma counter. Maximal binding was determined from tubes containing no cold antibodies.

IL-2 Bioassay

Biological activity of chLym-1/IL-2 preparations was determined by a standard IL-2-dependent T-cell proliferation assay (27). Two IL-2 standards were used. Carrier-free IL-2 was obtained from Hoffmann-La Roche, Inc. (Nutley, NJ). Roche IL-2 stock (7.4 µg/ml, specific activity ~45 x 10^4 IU/µl) was diluted to yield a stock solution containing 1 x 10^5 Biological Response Modifier Program units/µl (3 x 10^6 IU/µl). IL-2 was also obtained from Cetus (Emeryville, CA) at a specific activity of 18 x 10^4 IU/ml and diluted with water according to the manufacturer's instructions. The growth of the IL-2-dependent murine T-cell line, CTLL-2, was used to determine the amount of IL-2 bioactivity in a sample. Briefly, serially diluted samples and standards were incubated with 4 x 10^5 CTLL-2 cells in triplicate for 15 h at 37°C in 96-well flat-bottom microtiter plates. The cells were then pulsed with 0.5 µCi of [3H]thymidine for 6 h, and the samples were harvested and counted.

Cytotoxicity Assays

ADCC was performed using the chromium release method described previously (21). Briefly, Raji cells were labeled for 2 h at 37°C with 250 µCi Na251CrO4 in RPMI 1640. The cells were subsequently washed and added to 96-well V-bottomed microtiter plates. Different antibody preparations (chLym-1/IL-2, chLym-1, muLym-1, or muB72.3) were added in triplicate to individual wells at various antibody concentrations (10–0.001 µg/ml). Fresh peripheral blood mononuclear effector cells from healthy human donors were immediately added at various effector:target cell ratios to the assay plates. The plates were incubated for 4 h at 37°C and centrifuged. Supernatants were then harvested, and the radioactivity was measured in a gamma counter. Maximum release was obtained by lysing the Raji cells with 10% SDS. Spontaneous release was detected in the wells that contained only target cells without antibody. The percentage of specific lysis or cytotoxicity was calculated as:

\[ \% \text{specific lysis} = \frac{\text{Experimental release} - \text{spontaneous release}}{\text{Maximal release} - \text{spontaneous release}} \times 100 \]

CMC was determined using an assay similar to that described above for ADCC, except that instead of effector cells, complement was added to the labeled Raji cells. Fresh human serum was used as a source of complement. Serum was added to each well, and the cells were incubated at 37°C for 4 h in the presence of different antibody preparations at various concentrations. Supernatants were harvested and counted. The percentage of specific lysis or cytotoxicity was calculated as for ADCC. ANOVA was used for statistical analysis of these data.

IEF of Purified MAbs

IIFs were determined by IEF in a Bio-Rad model 111 Mini IEF cell through a pH gradient constructed with a mixture of BioLyte ampholytes (Bio-Rad) at concentrations of 1.2% 3/10 ampholyte and 0.8% 5/8 ampholyte according to protocols provided by Bio-Rad. Purified antibodies were applied directly to the gel, and IEF was carried out under constant voltage. IEF standards (Bio-Rad) were included in each run to determine the pl of the samples. IEF gels were fixed and stained with Coomassie blue R-250 and dried overnight.

Determination of Antibody Avidity

To determine the avidity constant (K_a) of chLym-1/IL-2, a live cell RIA was performed using a directly labeled antibody preparation by the method of Frankel and Gerhard (28). Each experimental variable was run in duplicate. Washed Raji cell suspensions containing 10^6 cells/ml were added to test tubes, and the cells were incubated with 10–110 ng of 125I-labeled chLym-1/IL-2 in
200 µl of PBS for 1 h at room temperature with constant shaking. The cells were then washed three times with PBS containing 1% BSA to remove unbound antibody and counted in a gamma counter. The amount of bound antibody was then determined by the remaining cell-bound radioactivity (cpm) in each test tube and the specific activity (cpm/ng) of the radiolabeled MAb. Scatchard plot analysis was used to obtain the slope of the data. From the slope, the equilibrium, or avidity constant $K_a$ was calculated by the equation $K_a = -$(slope/n), where n is the valence of the antibody (2 for IgG).

**Pharmacokinetic and Biodistribution Studies**

Six-week-old non-tumor-bearing BALB/c mice were used to determine pharmacokinetic clearance of chLym-1 and chLym-1/IL-2. Different groups of BALB/c mice (n = 5) were given i.p. injections of radiolabeled MAbs (30–40 µCi/mouse). The whole-body activity at injection and at selected times thereafter was measured with a CRC-7 microdosimeter (Capintec Inc., Pittsburgh, PA). The data were analyzed, and half-lives were determined using the RSTRIP pharmacokinetic program (MicroMath, Inc., Salt Lake City, UT).

To determine tissue biodistribution of chLym-1/IL-2, female athymic nude mice (BALB/c background) were obtained at approximately 6 weeks of age. The mice were irradiated with 400 rads from a cesium source and 3 days later were injected with a 0.2-ml inoculum containing $4 \times 10^7$ Raji cells and $4 \times 10^8$ human fetal lung fibroblast feeder cells s.c. in the left thigh. The tumors were grown for 12–18 days until they reached $0.5\text{cm}^3$ in diameter. Within each group of mice (n = 5), individual mice were injected i.v. with a 0.2-ml inoculum containing 100 µCl/10 µg of $^{125}$I-labeled chLym-1/IL-2. All animals were sacrificed by sodium pentobarbital overdose at 24 and 72 h postinjection, and various organs, blood, and tumor were removed and weighed. The samples were then counted in a gamma counter. For each mouse, data were expressed as tumor:organ ratio (cpm per gram tumor:cpm per gram organ) and percent injected dose/gram (%ID/g). From these data, the mean and SD were calculated for each group.

**Enhancement of Vascular Permeability**

To demonstrate that pretreatment with chLym-1/IL-2 leads to increased vascular permeability at the tumor site and increased uptake of macromolecules, the following studies were undertaken. Raji tumor-bearing mice generated as above were injected i.v. with 50 µg of chLym-1/IL-2 followed 3 h later by a 0.2-ml inoculum containing 100 µCl/10 µg of $^{125}$I-labeled chLym-1 or $^{125}$I-labeled mouse albumin. Twenty-four (mouse albumin) or 72 h (chLym-1) hr later, biodistribution studies were performed as above. Student’s t test was used for statistical analysis of these data.

**RESULTS**

**Construction, Expression, and Purification of chLym-1/IL-2.**

The human γ1 sequence of the chLym-1 heavy chain was mutated by PCR using primers to generate a unique SpeI restriction endonuclease site immediately before the γ1 stop codon. A PCR fragment containing the human IL-2 gene preceded by a five amino acid linker was then inserted into the SpeI site (Fig. 1). The fusion gene was digested with BglII and BamHI and cloned into the BamHI site of the BV transfer vector containing the chLym-1 light chain gene to generate the complete vector pBVchLym-1/IL-2. The fusion genes were expressed in High Five insect cells, and the protein product was purified from the supernatant by protein A affinity chromatography. The chLym-1/IL-2 expressed in this system was correctly processed and assembled into the expected immunoglobulin monomer as demonstrated by SDS-PAGE in reducing gels with the observation of two well-defined bands at 25 and 66 kDa, corresponding to the molecular weights of immunoglobulin light chain and heavy chain plus human IL-2 (Fig. 2). IEF of chLym-1/IL-2 gave a pI of 8.4, whereas chLym-1 focused into a single band of pI 8.7 (data not shown).

**Immunobiochemical Analysis.**

Purified chLym-1/IL-2 was evaluated for its immunoreactivity with the target antigen of muLym-1 by assaying its binding to antigen-bearing Raji lymphoma cells. In a RIA, increasing concentrations of chLym-1/IL-2, muLym-1, or an irrelevant MAb (muLym-2) were evaluated for their ability to inhibit binding of $^{125}$I-labeled muLym-1 to Raji lymphoma cells. muLym-1 and Lym-2 served as positive and negative controls, respectively.

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biological activity of the IL-2 moiety, we assayed the ability of chLym-1/IL-2 to support IL-2-dependent T-cell proliferation. A bioassay with the IL-2-dependent CTLL-2 line was performed; chLym-1/IL-2 was assayed, along with IL-2 standards (Fig. 6). On a molar basis, chLym-1/IL-2 had an average of 25% of the activity required to produce 50% maximum proliferation of the IL-2-dependent cell line compared to recombinant IL-2 standards. At higher concentrations (e.g., >1 nM), maximum proliferation was achieved as evidenced by the plateau of the incorporation of [3H]thymidine into DNA.

In Vivo Pharmacokinetic and Tumor Binding Studies. Whole-body clearance studies were performed to establish pharmacokinetic differences between chLym-1/IL-2 and chLym-1. Normal non-tumor-bearing mice were injected with 125I-labeled chLym-1/IL-2, and the whole-body activity was determined at various time points by placing the mice in a microdosimeter. chLym-1/IL-2 cleared very fast and had a whole-body half-life of 11 h (Fig. 7). By comparison, the whole-body half-lives of chLym-1 and muLym-1 were 20 and 99 h, respectively. The faster clearance of chLym-1/IL-2 was apparent when its tumor uptake was assayed by biodistribution in tumor-bearing nude mice (Table 1). Compared to chLym-1, the overall uptake of chLym-1/IL-2 was significantly lower in vivo (2.74 ± 0.16% and 2.82 ± 0.18% versus 4.34 ± 0.73% and 4.95 ± 0.32% ID/g at 24 h).

Cytotoxicity Studies. chLym-1/IL-2 and chLym-1 were evaluated for their ability to mediate ADCC and CMC in standard 51Cr release assays against Raji target cells. chLym-1/IL-2 mediated approximately 2-fold higher ADCC than chLym-1 at the maximal MAb concentration of 1 µg/ml when the effector:target cell ratio was held constant at 100:1 (Fig. 4a). When the antibody concentration was held constant at 1 µg/ml and the effector:target cell ratio varied, maximal cytotoxicity of 85% was obtained at a ratio of 200:1 (Fig. 4b). chLym-1/IL-2 was also evaluated for its ability to induce CMC, and it induced 67% cytotoxicity at a MAB concentration of 1 µg/ml, approximately the same as that induced by chLym-1 (Fig. 5).

IL-2 Bioactivity of chLym-1/IL-2. Because our initial hypothesis was that a genetically engineered fusion protein would maintain

Fig. 4. ADCC activity of chLym-1/IL-2. MAbs were cultured with 51Cr-labeled Raji target cells and human peripheral blood mononuclear cell effectors at varying concentrations of MAB and E:T ratios as indicated. A, E:T ratio was held constant at 100:1, and the MAB concentration was increased from 0.001 to 10 µg/ml. B, the MAB concentration was held constant at 1 µg/ml, and the E:T ratio was increased from 10:1 to 200:1. At all ratios: *, significant increase over muLym-1 (P < 0.001); **, significant increase over chLym-1 (P = 0.001). Human IgG and murine B72.3 served as negative controls.

Fig. 5. CMC activity of chLym-1/IL-2. MAbs were cultured with 51Cr-labeled Raji target cells and fresh human serum as a complement source at varying MAB concentrations as indicated. Human IgG and murine B72.3 served as negative controls.

Fig. 6. Biological activity of chLym-1/IL-2 as determined by the ability to support the proliferation of CTLL-2 cells. Serial dilutions of samples and recombinant IL-2 standards were incubated with 4 × 10^6 CTLL-2 cells in triplicate for 15 h at 37°C. The cells were pulsed with 0.5 µCi of [3H]thymidine for 6 h, and the samples were harvested and counted.

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Fig. 7. Whole-body pharmacokinetic clearance of muLym-1, chLym-1, and chLym-1/IL-2 in non-tumor-bearing mice. Bars, SD.

(P = 0.001) and 72 h (P < 0.0001), respectively; the data for chLym-1 have been published previously (21). Tumor:organ ratios, indications of normal organ uptake, were considerably higher, however, with chLym-1/IL-2. Tumor:blood ratios, normally the lowest of all organs (indicating high uptake in blood), were 18:1 and 420:1 at 72 h for chLym-1 and chLym-1/IL-2, respectively. This lower normal organ uptake for chLym-1/IL-2 indicates that very little of this reagent is binding to normal tissues, where toxicity caused by the IL-2 moiety can occur.

Enhancement of Vasopermeability at the Tumor Site. chLym-1/IL-2 was assayed for its ability to induce vasopermeability at the tumor site and increase uptake of radiolabeled mouse albumin and chLym-1. Raji lymphoma-bearing nude mice were injected with 50 μg of chLym-1/IL-2 or chLym-1 and 3 h later injected with 125I-labeled mouse albumin. The mice were sacrificed 24 h later, and biodistribution analysis was performed. In this study, pretreatment with the vasoactive fusion protein increased the tumor uptake of radiolabeled mouse albumin 2.5-fold (Table 2).

In a second study, Raji lymphoma-bearing nude mice were injected with 50 μg of chLym-1/IL-2 fusion protein, followed 3 h later with 125I-labeled chLym-1 without pretreatment. Biodistribution analysis was performed after 3 days for both groups. The results of this study were consistent with those seen with the muLym-1/IL-2 chemical conjugate (Table 3), although the magnitude of the enhancement was less. Uptake of chLym-1 was increased from 4.95 ± 0.32% with no pretreatment to 6.53 ± 0.29% after chLym-1/IL-2 pretreatment, a 32% increase in tumor uptake of radiolabeled antibody (P < 0.0001). As shown below, even more dramatic increases were noted in the tumor:organ ratios by chLym-1/IL-2 pretreatment with values now more than 1000:1 in some organs.

DISCUSSION

In this study, a genetically engineered fusion protein consisting of the chimeric MAb Lym-1 and IL-2 has been generated that retains both tumor targeting and cytokine activities. The insect BV expression system was used to facilitate the rapid generation and characterization of the fusion protein because recombinant proteins can be attained much more quickly in this system than in a mammalian expression system. Moreover, the production of recombinant proteins in insect cells allows for correct processing, folding, and glycosylation (29). However, because the BV produces a lytic infection that kills the infected cells after a short period of time and therefore results in relatively low expression levels (5—10 μg/ml), it is not well suited for large-scale production. To produce high levels of chLym-1/IL-2 for clinical evaluation, the glutamine synthetase gene amplification system is being used for expression from myeloma cells (30). This mammalian expression system can enable large-scale expression levels up to 500 μg/ml.

Biochemical analysis of the chLym-1/IL-2 fusion protein demonstrates the presence of two IL-2 moieties per antibody molecule as evidenced by the increase in molecular weight over native chLym-1 (Fig. 2). The IL-2 moiety is present at the COOH terminus of the immunoglobulin heavy chain following a short peptide linker that enables proper folding of the IL-2. Extensive analysis of the fusion protein shows that the antibody binding sites successfully compete with chLym-1 and muLym-1 in Raji binding assays and that there is little change in the affinity binding constant of the fusion protein when compared with native antibody. The functional properties of the IL-2 component of the fusion protein were demonstrated by several methods, including bioassay, ADCC studies, and vascular permeability determinations in tumor-bearing nude mice. The results of these studies demonstrate that the fusion protein retains the proliferation, cytotoxic activation, and vasopermeability activities of IL-2. Specifically, the fusion protein is able to support in vitro T-cell proliferation at one-fourth the level of recombinant IL-2 and produces a 2- and 4-fold higher ADCC response when compared to chLym-1 and muLym-1, respectively. Like chLym-1, the fusion protein shows a markedly decreased whole-body half-life compared to muLym-1 due to the presence of human sequences. Despite a whole-body half-life that is 9-fold shorter than muLym-1 (11 h versus 99 h), biodistribution analyses

Table 1: Biodistribution of chLym-1/IL-2 in nude mice bearing Raji lymphoma tumors

<table>
<thead>
<tr>
<th>Organ</th>
<th>% Injected dose/gram</th>
<th>Tumor:organ ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>24 h</td>
<td>72 h</td>
</tr>
<tr>
<td>Blood</td>
<td>0.08 (0.04)*</td>
<td>0.02 (0.01)</td>
</tr>
<tr>
<td>Skin</td>
<td>0.06 (0.03)</td>
<td>0.02 (0.01)</td>
</tr>
<tr>
<td>Muscle</td>
<td>0.06 (0.02)</td>
<td>0.02 (0.01)</td>
</tr>
<tr>
<td>Bone</td>
<td>0.05 (0.04)</td>
<td>0.03 (0.01)</td>
</tr>
<tr>
<td>Heart</td>
<td>0.12 (0.07)</td>
<td>0.03 (0.01)</td>
</tr>
<tr>
<td>Lung</td>
<td>0.16 (0.06)</td>
<td>0.05 (0.03)</td>
</tr>
<tr>
<td>Liver</td>
<td>0.07 (0.04)</td>
<td>0.01 (0.01)</td>
</tr>
<tr>
<td>Spleen</td>
<td>0.04 (0.04)</td>
<td>0.02 (0.01)</td>
</tr>
<tr>
<td>Pancreas</td>
<td>0.30 (0.47)</td>
<td>0.04 (0.02)</td>
</tr>
<tr>
<td>Stomach</td>
<td>0.07 (0.08)</td>
<td>0.00 (0.01)</td>
</tr>
<tr>
<td>Intestine</td>
<td>0.64 (0.19)</td>
<td>0.28 (0.07)</td>
</tr>
<tr>
<td>Kidney</td>
<td>2.74 (0.16)</td>
<td>2.82 (0.18)</td>
</tr>
<tr>
<td>Tumor</td>
<td>114.60 (52.56)</td>
<td>4.54 (1.19)</td>
</tr>
</tbody>
</table>

* Mean (SD).
There is a great body of literature that describes the therapeutic potential of IL-2 in the treatment of cancer (33, 34). Researchers, however, have only just begun to explore the use of IL-2 in conjunction with MAbs. Previous studies by Gill et al. (35) using free IL-2 and muLym-1 and other MAbs (36–39) have demonstrated that the combination of these reagents produces a more potent ADCC reaction both in vitro and in vivo, causing significant retardation of tumor growth. Moreover, more sophisticated experiments using gene transfer have shown that tumor cells engineered to secrete functional IL-2 selectively inhibit tumor cell growth in susceptible hosts. Tumor models used to demonstrate the effectiveness of this approach include mouse mammary cells (40), mouse neuroblastoma cells (41), mouse thymoma cells (42), mouse Lewis lung carcinoma cells (43), human melanoma (44), and human acute leukemia cells (45). Although these methods are presently impractical in the clinical setting, they do demonstrate the importance of targeting IL-2 to the tumor to obtain optimal therapeutic efficacy while producing minimal toxicity. An alternative approach is the generation of recombinant antibody-IL-2 fusion proteins using the pan-carcinoma MAb L6 (46), the anti-GD2 chimeric MAb ch14.18 (47–49), an antilysozyme single-chain MAb (50), the antihuman epidermal growth factor receptor chimeric MAb ch225 (51), and the anticolon carcinoma TAG-72 single chain MAb (52, 53). As in our experiments, these investigators were able to demonstrate retention of IL-2 cytokine activity, which could be targeted to the tumor in appropriate animal models. As stated by Naramura et al. (51), one of the important rationales for producing these fusion proteins is the ability to deliver relatively larger amounts of IL-2 to the tumor site in such a way as to minimize the toxicity seen with systematically administered IL-2. Indeed, the major reason for developing these novel reagents is to harness the cytokine activity of potent immunostimulatory molecules close to their site of action. In our study, we intend to use an additional property of IL-2, i.e., its vasopermeability effects, to increase the amount of therapeutic antibody or drug targeting the tumor. To test the cytokine functionality of the chLym-1/IL-2 fusion protein, studies will have to be conducted in clinical trials because the Lym-1 antigen is not present in animal lymphomas, and an appropriate animal tumor model is therefore not available. Like Gillies et al. (47, 48), we believe that antibody fusion proteins represent a new approach for the delivery of potent cytokines to the tumor. As demonstrated by our studies, these reagents may be used additionally to enhance antibody uptake in tumors to improve their therapeutic potential. Moreover, a two-pronged attack represented by the delivery of both a radionuclide and IL-2 may produce better therapeutic responses than treatments using either a radionuclide or the cytokine alone.

<table>
<thead>
<tr>
<th>Organ</th>
<th>No pretreatment</th>
<th>Pretreatment</th>
<th>Tumor:organ ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blood</td>
<td>0.36 (0.10)</td>
<td>0.02 (0.01)</td>
<td>14.79 (4.13)</td>
</tr>
<tr>
<td>Skin</td>
<td>0.19 (0.05)</td>
<td>0.03 (0.01)</td>
<td>27.00 (6.79)</td>
</tr>
<tr>
<td>Muscle</td>
<td>0.05 (0.02)</td>
<td>0.01 (0.00)</td>
<td>99.53 (32.45)</td>
</tr>
<tr>
<td>Bone</td>
<td>0.08 (0.04)</td>
<td>0.02 (0.01)</td>
<td>67.25 (25.84)</td>
</tr>
<tr>
<td>Heart</td>
<td>0.15 (0.08)</td>
<td>0.01 (0.00)</td>
<td>44.92 (26.25)</td>
</tr>
<tr>
<td>Lung</td>
<td>0.47 (0.66)</td>
<td>0.02 (0.01)</td>
<td>16.40 (8.51)</td>
</tr>
<tr>
<td>Liver</td>
<td>0.14 (0.02)</td>
<td>0.04 (0.01)</td>
<td>36.15 (6.06)</td>
</tr>
<tr>
<td>Spleen</td>
<td>0.16 (0.08)</td>
<td>0.02 (0.01)</td>
<td>37.54 (16.97)</td>
</tr>
<tr>
<td>Pancreas</td>
<td>0.08 (0.02)</td>
<td>0.01 (0.00)</td>
<td>68.06 (19.76)</td>
</tr>
<tr>
<td>Stomach</td>
<td>0.09 (0.03)</td>
<td>0.02 (0.01)</td>
<td>62.09 (19.53)</td>
</tr>
<tr>
<td>Intestine</td>
<td>0.06 (0.02)</td>
<td>0.01 (0.00)</td>
<td>93.03 (33.58)</td>
</tr>
<tr>
<td>Kidney</td>
<td>0.25 (0.05)</td>
<td>0.13 (0.05)</td>
<td>20.52 (4.36)</td>
</tr>
<tr>
<td>Tumor</td>
<td>4.95 (0.32)</td>
<td>6.53 (0.29)</td>
<td>420.36 (253.13)</td>
</tr>
</tbody>
</table>

a Mean (SD).

b Significant increase in uptake ($P < 0.0001$).
REFERENCES

A Chimeric Lym-1/Interleukin 2 Fusion Protein for Increasing Tumor Vascular Permeability and Enhancing Antibody Uptake

Peisheng Hu, Jason L. Hornick, Michelle S. Glasky, et al.


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