Identification of a Protein Fragment of Interleukin 2 Responsible for Vasopermeability

Alan L. Epstein, Myra M. Mizokami, Jiali Li, Peisheng Hu, Leslie A. Khawli

Background: The cytokine interleukin 2 (IL-2) is involved in the activation of T cells and has been shown to play a central role in cancer immunotherapy. The full therapeutic potential of IL-2, however, has not been realized because of its dose-limiting systemic toxicity. We sought to identify a region of IL-2 that is responsible for the induction of vasopermeability (leaky tumor endothelium), a property associated with the toxicity of the molecule. Methods: Intact IL-2 or overlapping synthetic peptides of IL-2 that were chemically conjugated to tumor-targeting monoclonal antibodies (TNT-1 or Lym-1) were injected into groups of mice (n = 4) that had previously been xenotransplanted with human tumor cells (ME-180 cervical carcinoma and Raji lymphoma). Two hours later, mice received intravenous injections of radiolabeled tracer antibody, and 3 days later they were subjected to biodistribution analysis to measure the ability of each immunoconjugate to enhance tumor uptake of the tracer antibody (i.e., vasopermeability activity). The cytokine activity of the immunoconjugates was determined by assaying their ability to promote the proliferation of a mouse IL-2-dependent cell line. Results: Pretreatment of mice with an antibody/IL-2 immunoconjugate resulted in an approximately fourfold increase in radiolabeled tracer antibody uptake in the xenograft tumor as compared with uptake in mice injected with antibody alone. One synthetic fragment consisting of amino acids 22–58 contained 100% of the vasopermeability activity of IL-2 and was designated permeability-enhancing peptide (PEP). PEP had vasopermeability activity only when conjugated to a tumor-targeting antibody, had maximal activity as a dimer, and was devoid of cytokine activity. Conclusions: The identification of PEP should aid in the discovery of ways to decrease the toxicity of IL-2. Moreover, PEP is a promising candidate for the generation of agents that can enhance the delivery of antibodies and drugs to tumors. [J Natl Cancer Inst 2003;95:741–9]

Interleukin 2 (IL-2), a 15 000-kd protein produced by helper T lymphocytes, occupies a central role in the augmentation of cell-mediated immune responses. Its cytokine functions include stimulating the proliferation of T lymphocytes (1) and promoting nonspecific tumor killing by activated macrophages, lymphokine-activated killer cells (2), and tumor-infiltrating lymphocytes (3). In addition to its cytokine activity, IL-2 contains an analgesic domain (4) and induces vascular permeability when administered systemically by causing the efflux of intravascular fluids to extravascular spaces, a phenomenon known as capillary leak syndrome (5–8). Because of its stimulatory activity on a variety of immune cell types, IL-2 has been studied extensively in the augmentation of the innate immune response to malignant disease. Indeed, IL-2 has been shown to be clinically effective for the treatment of both malignant melanoma and renal-cell carcinoma (9,10). In addition to being investigated for the treatment of cancer, IL-2 also has been used as a component of antibody fusion proteins to target tumors (11–13) and is being tested as a method for increasing the number of circulating T cells in acquired immunodeficiency syndrome (AIDS) patients successfully treated by multidrug therapy (14). The clinical utility of IL-2, however, has been limited by its substantial side effects, especially capillary leak syndrome (15,16), which affects as many as 65% of patients and can lead to cessation of therapy (17). It is therefore important to elucidate the underlying mechanism(s) of toxicity produced by systemically administered IL-2 so that strategies for reducing or eliminating its toxicity can be developed.

Human IL-2 is a 133-amino-acid globular protein that is structurally similar to other cytokines, including IL-4 and granulocyte-macrophage colony-stimulating factor (18). Structural studies of IL-2 show that it is composed of four major amphipathic alpha helices that are arranged in an antiparallel fashion, with the hydrophobic faces making a stable hydrophobic core (19,20). A disulfide bond between amino acids 58 and 105 is essential for the stability of the tertiary structure and biologic activity of IL-2 (21). Site-directed mutagenesis studies (22) indicate that the loss of this disulfide bond or minor changes in the primary or secondary structure of IL-2 abrogate its cytokine activity. There are several forms of IL-2 receptors that differ in their affinities for IL-2: a low-affinity receptor consisting of an alpha chain, an intermediate-affinity receptor consisting of beta and gamma chains, and a high-affinity receptor consisting of all three chains (23).

Our laboratory has previously described experiments to harness the permeability-inducing property of IL-2 to induce localized vasopermeability at the tumor site by covalently linking it to a tumor-specific monoclonal antibody (24,25). The generation of a leaky tumor endothelium by pretreatment with antibody/IL-2 immunoconjugates produces a two- to fourfold increase in tumor uptake of antibodies and chemotherapy drugs (24). The chemical procedures used to link IL-2 to antibodies destroy the cytokine activity of IL-2 but do not affect its vasopermeability activity. However, we (11,24,25) and others (12,26–28) have shown that fusing IL-2 to the carboxyl terminus of the heavy chain of antibodies by genetic engineering preserves the cytokine activity of the molecule.

Taken together, these findings indicate that the vasopermeability activity of IL-2 is more stable than its cytokine properties. Consequently, we investigated whether the vasopermeability activity of IL-2 could be mapped to a specific region of the protein.
by generating contiguous and overlapping synthetic peptide fragments of the molecule and then testing them for vasopermeability activity. If the vasopermeability and cytokine effects of IL-2 can be separated, it may be possible to use a synthetic peptide containing the vasopermeability activity to enhance the delivery of drugs or other macromolecules to tumors or other antibody-targeted tissues. Conversely, a version of IL-2 that has lost vasopermeability activity but retains cytokine activity would have less systemic toxicity than intact IL-2 and could therefore have improved therapeutic value.

**MATERIALS AND METHODS**

**Reagents**

All chemicals were purchased from Sigma Chemical Co. (St. Louis, MO) except where noted. All solvents were of analytical grade and were used as purchased. 125I was obtained as sodium iodide in 0.05 N sodium hydroxide solution (ICN Biomedicals, Irvine, CA). Radioactivity in samples was measured using either a 1282 CompuGamma counter (LKB Instruments, Pleasant Hill, CA) or a CRC-7 dose calibrator (Capintec, Pittsburgh, PA).

Instant thin-layer chromatography (ITLC) was carried out on silica gel-impregnated fibers (No. 61886; Gelman Sciences, Ann Arbor, MI). Gel exclusion chromatography was performed with Sephadex G-10, G-25, and G-100 columns (Sigma). Flash chromatography was carried out as previously described (29) using Kieselgel 60, 230–400 mesh (No. 9385; E. Merck, Darmstadt, Germany). Fast protein liquid chromatography (FPLC) separations were performed at room temperature using a system equipped with two P-500 solvent pumps, an MV-8 motor valve, a single-path UV monitor (280 nm), an LCC-500 autosampler, and an REC-482 dual-pen chart recorder (Pharmacia, Piscataway, NJ). Buffers used in FPLC procedures were filtered through 0.22-μm Nalgene disposable filter units before use.

Proton (1H) nuclear magnetic resonance (NMR) was recorded on a Hitachi PerkinElmer R-24 60-MHz instrument. NMR sample concentrations were approximately 10% (w/v) in the indicated solvent. Chemical shifts (in parts per million) are reported downfield (δ) relative to an internal tetramethylsilane standard.

Murine monoclonal antibodies Lym-1 (immunoglobulin G2a) and TNT-1 (immunoglobulin G2a) were obtained from Peregrine Pharmaceuticals (Tustin, CA). Lym-1 is directed against a variant of the HLA-DR10 antigen, which is expressed on the cell surface of human B lymphocytes and malignant lymphomas (30). TNT-1 recognizes an epitope of the DNA/histone complex that is retained in the nuclei of degenerating mammalian cells (31). Protein concentrations of the antibody preparations were estimated by optical spectroscopy at 280 nm. Human recombinant IL-2 was obtained from Hoffman-La Roche (Nutley, NJ). Human serum albumin (HSA) was obtained from Sigma.

For in vivo experiments, the Raji Burkitt’s lymphoma cell line and the ME-180 human cervical carcinoma cell line were used as previously described (32). Both cell lines were grown in RPMI-1640 medium (Life Technologies, San Diego, CA) containing 10% fetal calf serum (HyClone, Logan, UT), penicillin G at 100 U/mL, and streptomycin sulfate at 100 mg/mL and cultured at 37 °C in a humidified 5% CO2 incubator.

**Synthesis of Human IL-2 Peptide Fragments**

Peptide fragments corresponding to portions of human IL-2 were synthesized by the Merrifield method (33) using a one-column peptide synthesizer (model 430A; Applied Biosystems, Foster City, CA) at the Microchemical Core Facility of the Norris USC Comprehensive Cancer Center (Los Angeles, CA). Fragments of 16–46 amino acids, representing all but the first 22 amino acids of IL-2, were prepared. The protected peptides were assembled by solid-phase synthesis and then cleaved by trifluoroacetic acid (34,35), purified by gel filtration on a Sephadex G-10 column in 30% acetic acid, and lyophilized. A list of the peptide fragments of IL-2 used in these experiments is provided in Table 1.

**Preparation of IL-2 Conjugates**

**Antibody/IL-2.** Lym-1 and TNT-1 antibodies and HSA were coupled to IL-2 as previously described (36). Radiolabeled IL-2 was added in trace amounts to the reaction mixture to confirm its binding to the antibody or HSA. To produce radiolabeled IL-2, lypophilized IL-2 was dissolved in water to a final concentration of 2 mg/mL. Fifty microliters of this solution (i.e., 100 μg of IL-2), 100 μCi of carrier free 125I, and 5 μL of chloramine T (10 mg/mL in water) were added to 100 μL of phosphate-buffered saline (PBS; pH 7.2), and the reaction was allowed to proceed for 1 minute at room temperature. The reaction was then quenched by the addition of 100 μL of anion exchange resin (AG1-X8; Bio-Rad Laboratories, Richmond, CA) in PBS. After 1 minute the suspension was withdrawn and the resin was removed by low-speed centrifugation (400g for 5 minutes at room temperature) using a Spin-X centrifuge unit (Costar, Cambridge, MA).

The coupling reaction was initiated by adding 1 mg of IL-2 (500 μL of the 2 mg/mL solution) to 500 μL of a solution containing 10 mg/mL of antibody in PBS, 1-ethyl-3-[3-dimethylamino-1-propanoyl]-pyridinium 3-oxide tetrafluoroborate (PEP) (500 Ci of carrier-free 125I, and 5 μL of chloramine T (10 mg/mL in water) were added to 100 μL of phosphate-buffered saline (PBS; pH 7.2), and the reaction was allowed to proceed for 1 minute at room temperature. The reaction was then quenched by the addition of 100 μL of anion exchange resin (AG1-X8; Bio-Rad Laboratories, Richmond, CA) in PBS. After 1 minute the suspension was withdrawn and the resin was removed by low-speed centrifugation (400g for 5 minutes at room temperature) using a Spin-X centrifuge unit (Costar, Cambridge, MA).

**Table 1. Vasopermeability activity of IL-2 synthetic peptide fragments and immunoconjugates**

<table>
<thead>
<tr>
<th>Amino acid sequence of IL-2 fragments</th>
<th>Fragment/immunoconjugate</th>
<th>Vasopermeability as % Lym-1/IL-2 (95% CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>3A</td>
<td>Lym-1/3A</td>
<td>0 (0.2 to 0.7)</td>
</tr>
<tr>
<td>3B</td>
<td>Lyr-1/3B</td>
<td>0 (0.3 to 0.6)</td>
</tr>
<tr>
<td>3C</td>
<td>Lyr-1/3C</td>
<td>50 (48 to 52)</td>
</tr>
<tr>
<td>3D</td>
<td>Lyr-1/3D</td>
<td>0 (0.3 to 0.6)</td>
</tr>
<tr>
<td>3E</td>
<td>Lyr-1/3E</td>
<td>50 (46 to 53)</td>
</tr>
<tr>
<td>3F</td>
<td>Lyr-1/3F</td>
<td>0 (0.2 to 0.5)</td>
</tr>
<tr>
<td>3G</td>
<td>Lyr-1/3G</td>
<td>0 (0.3 to 0.4)</td>
</tr>
<tr>
<td>3H</td>
<td>Lyr-1/3H</td>
<td>0 (0.2 to 0.6)</td>
</tr>
<tr>
<td>IL-2</td>
<td>1–133</td>
<td>0 (0.3 to 0.7)</td>
</tr>
<tr>
<td>IL-2</td>
<td>1–133</td>
<td>0 (0.1 to 0.6)</td>
</tr>
<tr>
<td>IL-2</td>
<td>1–133</td>
<td>75 (72 to 79)</td>
</tr>
</tbody>
</table>

*Raji lymphoma-bearing mice were first injected intravenously with 30 μg of peptide or IL-2 and then, 2 hours later, with 125I-Lym-1. After 72 hours, mice were subjected to biodistribution analysis to determine vasopermeability of tumor vessels. Data are presented as percentages of the vasopermeability induced by the Lyr-1/IL-2 immunoconjugate: IL-2 = interleukin 2; CI = confidence interval; PEP = permeability-enhancing peptide.*
Antibody/peptide conjugates. Radiolabeled synthetic peptide fragments of IL-2 were also added in trace amounts to antibody/peptide conjugation reactions to confirm the binding of each fragment to the antibody. For the radiolabeling procedure, lyophilized fragments were dissolved in 10% aqueous ethanol to a final concentration of 1 mg/mL. One hundred microliters of antibody and 100 μCi of Na125I in 0.1 N NaOH previously neutralized with 100 μL of 0.1 M acetic acid. The mixture was stirred vigorously, and two iodo-beads (Pierce) were added. After 1 hour at room temperature, the mixture was washed with 100 μL of 10% aqueous ethanol. The washes were added back to the reaction mixture, and the solution was purified on a short Sephadex G-10 column eluted with PBS, pH 7.4. The purity of the radiolabeled fragments was determined by analytical ITLC. ITLC strips (2 × 20 cm) were activated by heating at 110 °C for 15 minutes just before use, spotted with 1 μL of the sample, air dried, and developed with a solution of methanol and water (80:20 v/v) for approximately 12 cm, air dried again, and placed in a scintillation counter to determine peptide-bound and unbound radioactivity. In this system, free iodine migrated with the solvent, whereas labeled peptide fragments remained near the origin. For all IL-2 peptides, more than 90% of the radioactivity was associated with each fragment.

The reactions to couple the individual peptide fragments to the Lym-1 or TNT-1 antibody were initiated by combining the peptide fragment with the antibody, EDC, and sulfo-NHS in a 1:2:5 (w/w) ratio by weight in a total volume of 0.6 mL in PBS. The reactions were incubated overnight at 4 °C. The peptide-coupled antibody was chromatographed on a Sephadex G-100 column calibrated with blue dextran. From the antibody concentration and radioactivity, we calculated that approximately two molecules of IL-2 had bound to each antibody molecule. These immunoconjugates retained a minimum of 75% of the binding reactivity of the unmodified antibody, as determined by a fixed-cell binding assay (30,37).

Antibody/permeability-enhancing peptide dimer. The dimeric form of one of the IL-2 peptide fragments, 3D (subsequently referred to as permeability-enhancing peptide (PEP); see the “Results” section) was prepared by inducing the formation of a disulfide bond involving the cysteine at position 58 (Fig. 1, A). This was done by producing the thiol form of PEP by treatment with 10 mM 2-mercaptoethanol for 30 minutes followed by gel filtration on a Sephadex G-10 column equilibrated with 0.1 M sodium phosphate (pH 6.8). NaOH (5 M) was added until the solution reached pH 9.0, and the mixture was then incubated for 16 hours at room temperature (Fig. 1, A). The desired peptide dimer was purified from the reaction mixture by gel filtration on a Sephadex G-25 column equilibrated with 0.1 M phosphate buffer (pH 7.4). The yield of PEP dimer was approximately 90% under these conditions, without the formation of high-molecular-weight species. The PEP dimer was coupled to antibody using the conditions described above and was found to have approximately the same conjugation yield as the other peptides.

Antibody/PEP–phenylmaleimide monomer. Synthesis of N-phenylmaleimide. To prevent PEP from dimerizing, the thiol group on Cys-58 was modified by attaching an N-phenylmaleimide moiety. As depicted in Fig. 1, B, N-phenylmaleimide was synthesized by a modified procedure (38). Briefly, maleic anhydride (1.33 g, 13.6 mmol) was dissolved in 15 mL of toluene. Aniline (1.3 g, 13.9 mmol) dissolved in 20 mL of toluene was then added dropwise to this solution over a 20-minute period. The reaction mixture was stirred for 45 minutes at room temperature after which it was cooled in an ice-water bath. The precipitated product, N-phenylmaleamic acid, was collected by filtration, washed with hexane, and dried overnight (2.1 g yield).

1H NMR (Me2SO-d6, δ) analysis showed 10.3 (1H, singlet, OH); 7.7–7.8 (5H, multiplets, 5 aryl CH); 6.4 (2H, doublet of doublets,
COCH = CHCO). The N-phenylmaleimide acid (2.0 g, 10 mmol) was mixed with 15 mL of acetic anhydride. Sodium acetate (2.0 g) was then added, and the solution was stirred at 120°C until a brown precipitate formed. The precipitate was filtered and evaporated to dryness by vacuum using a standard rotovap apparatus, and 2 g of the residue was dissolved in 5 mL of diethyl ether. The ether mixture was filtered, and the filtrate was again evaporated to dryness. The residue was then applied to a flash chromatography column (30 × 200 mm) of Kieselgel 60 and eluted with 500 mL of ethyl acetate/hexane (1:3 v/v) in 50 fractions. Fractions 25–40 were combined to provide pure N-phenylmaleimide (1.5-g yield). ITLC analysis using ethyl acetate/hexane (1:3 v/v) showed an Rf value of 0.45. 1H NMR (CDCl3, δ) analysis showed 7–7.8 (5H, multiplets, 5 aryl CH); 6.8 (2H, singlet, COCH = CHCO).

Reaction of PEP with N-phenylmaleimide and the formation of the antibody/PEP monomer immunoconjugate. The N-phenylmaleimide was conjugated to PEP by adding a 2.5-fold molar excess of N-phenylmaleimide (in 15 μL of methanol) to 1 mg of PEP dissolved in 1 mL of 0.1 M citrate buffer (pH 6.0). The reaction was allowed to proceed for 30 minutes at 37°C, after which mercaptoethanolamine (15 mM final concentration) was added and the mixture was allowed to stand overnight to reduce any disulfide bonds that might have formed between the PEP monomers. The final reaction conjugate was purified by gel filtration on a Sephadex G-10 column and eluted with 0.1 M PBS (pH 7.2). As with the dimer, coupling of the PEP–phenylmaleimide monomer to the antibody was performed as described above for the other peptides and produced approximately the same conjugation yields.

Vasopermeability Studies

Radioiodination and analysis of antibodies. The Lym-1 and TNT-1 antibodies were radiolabeled with 125I using a modified chloramine T method (39), described above, so that they could be used as tracers in vasopermeability analysis. Radiolabeled antibodies were analyzed using an analytical ITLC system as described above. All preparations had the same radiochemical purity (98%). The immunoreactivity of radiolabeled Lym-1 and TNT-1 was determined by a fixed-cell radioimmunoassay procedure (37) which showed that both antibodies had an 80% binding reactivity after iodination.

Tumor models and biodistribution studies. Animal protocols were approved by both the Animal Safety and the Radiation Safety Committees at the Keck School of Medicine of the University of Southern California. The ME-180 human cervical carcinoma cell line was xenografted in the left flank of 6-week-old female athymic nude mice (Harlan Sprague Dawley, San Diego, CA) by subcutaneous injection of a 0.2-mL inoculum consisting of 1 × 107 cells. The tumors were grown for 3–4 weeks until they reached approximately 1 cm in diameter.

The Raji lymphoma cell line was used to produce xenograft tumors in 6-week-old female nude mice by subcutaneous injection of a 0.2-mL inoculum containing 2 × 107 Raji cells and 4 × 106 human fetal fibroblast feeder cells in the left flanks of the mice. Three days before injection, the mice were irradiated with 400 rads using a cesium irradiator to ensure a high rate of implantation. After 14–18 days, when the tumors had reached approximately 0.5 cm in diameter, separate groups of mice (four per group) were pretreated by intravenous injection with 30 μg of unlabeled antibody or antibody conjugate. After 2 hours, mice received 100-μL injections of 50 μCi of 125I-labeled Lym-1 or 20 μg TNT-1 antibody as tracer. In all experiments, the mice were killed 72 hours after injection, and the biodistribution of the tracer was analyzed as previously described (40). For each mouse tissue or organ, the data are expressed as the percentage of injected dose per gram of tissue and as tumor-to-organ ratios of injected radiolabeled antibody.

Cytokine Activity Studies

The proliferation of the murine IL-2-dependent indicator cell line, HT-2, was used to compare the cytokine activity of IL-2 (positive control), PEP, and antibody/PEP and antibody/IL-2 immunoconjugates. The HT-2 cell line, which was the generous gift of Dr. Minnie McMillan, Department of Microbiology, Keck School of Medicine of the University of Southern California, was maintained in RPMI-1640 medium containing 10% fetal calf serum, 1% antibiotics, and 20% T-STEM Culture Supplement (Collaborative Biomedical Products, Bedford, MA). The cells were passaged three times per week by diluting them 1:1 in new medium. Experiments were performed in triplicate on 2-day-old cultures of IL-2-starved cells. Briefly, HT-2 cells were washed three times with PBS, and 50 μL of cells (at 1 × 105 cells/mL) were plated in the wells of a 96-well plate containing serial dilutions of IL-2, antibody/PEP, or antibody/IL-2 to give a final volume of 100 μL/well. The plates were incubated for 72 hours in a humidified 37°C, 5% CO2 incubator, and 20 μL of CellTiter 96 AQueous One Solution (Promega, Madison, WI) was then added to each well. After 4 hours, cell proliferation was determined by reading the plates in a microtiter plate reader using a 490-nm filter.

Statistical Analysis

Statistical significance of differences in radiolabel tracer uptake in control and immunoconjugate-pretreated tumor-bearing mice was determined using the Wilcoxon rank-sum test (41).

RESULTS

Identification of Active Synthetic Peptide Fragments of IL-2

To identify the sequences within IL-2 that are responsible for its vasopermeability activity, we synthesized a series of overlapping or contiguous peptides (Table 1). Each peptide and native IL-2 was conjugated to Lym-1, and both the peptides and their respective immunoconjugates were subjected to biodistribution analysis to examine their ability to induce tumor vascular permeability and enhanced tracer uptake in Raji lymphoma-bearing nude mice. As shown in Table 1, an antibody/peptide conjugate derived from one synthetic peptide, 3D, had 100% (95% CI = 98% to 103%) of the vasopermeability activity of the Lym-1/IL-2 immunoconjugate. Consequently, it was renamed PEP. Three other antibody/peptide conjugates with peptides 3B, 3C, and 3F, all of which are either smaller fragments of PEP or overlap with PEP, had approximately half the vasopermeability activity of Lym-1/IL-2 in these assays. When the mice were pretreated with unconjugated 3D peptide and then injected with 125I-labeled Lym-1 tracer, Lym-1 uptake in the tumor was not enhanced (Table 1). By contrast, when the mice were pretreated with unconjugated IL-2, tumor uptake was enhanced (vasopermeability activity was approximately 75% that of the Lym-1/IL-2 immunoconjugate), suggesting that the native
IL-2 molecule is much more stable than the small synthetic peptide.

From the data in Table 1, it appears that the full sequence of amino acids 22–58 (peptide 3D) is required for maximal vasopermeability activity of PEP because antibody/peptide conjugates consisting of amino acids 37–58 (3B), 33–58 (3C), and 37–72 (3F) each had approximately 50% of the vasopermeability activity of the full peptide, and an antibody/peptide conjugate consisting of amino acids 22–38 (3E) had no activity. The complete amino acid sequence of PEP is as follows: Glu-Met-Ile-Leu-Asn-Gly-Ile-Asn-Asn-Tyr-Lys-Asn-Pro-Lys-Leu-Thr-Arg-Met-Leu-Thr-Phe-Lys-Phe-Tyr-Met-Pro-Lys-Ala-Thr-Glu-Leu-Lys-His-Leu-Gln-Cys. The DNA sequence of PEP has been added to GenBank (AF532913).

**In Vivo Analysis of PEP Immunoconjugates as Mediators of Increased Tumor Vascular Permeability in Tumor-Bearing Mice**

Nude mice bearing xenograft tumors from two different cell lines were pretreated for 2 hours with antibodies alone, antibody/IL-2 immunoconjugates, or antibody/PEP immunoconjugates. The effects of these pretreatments on tracer uptake in tumors were then determined by biodistribution analysis. TNT-1, which targets intracellular antigens accessible in permeable (i.e., dead) tumor cells (32), and its immunoconjugates were used in the ME-180 human cervical carcinoma/nude mouse tumor model. In complementary studies, Lym-1, which targets the HLA-DR10 cell-surface antigen (42,43), and its immunoconjugates were used in the Raji lymphoma-bearing nude mouse model. As shown in Fig. 2, A, mice pretreated with TNT-1 had 1.3% of the injected dose per gram of tissue of tracer accumulate in the tumor, whereas mice pretreated with TNT-1/IL-2 and TNT-1/PEP had 4.5% and 4.4% of the injected dose per gram of tissue, respectively, accumulate in the tumor. Similarly, mice pretreated with Lym-1 alone had only 1.4% of the tracer antibody accumulate in the tumor, whereas mice pretreated with Lym-1/IL-2 and Lym-1/PEP had 5.7% and 5.6% of the injected dose, respectively, accumulate in the tumor (Fig. 3, A). Thus, in both tumor models, conjugation of the antibody to IL-2 or PEP resulted in an approximately fourfold increase in radiolabeled tracer antibody uptake in the tumor. In addition, the specific targeting of the radiolabeled antibodies was increased after pretreatment with antibody/IL-2 or antibody/PEP immunoconjugates, as shown by the higher tumor/organ ratios that were achieved with the tracer antibody (Figs. 2, B, and 3, B). These results indicate that antibody-conjugated PEP has vasopermeability activity equivalent to that of conjugated intact IL-2 in two diverse tumor models.

**In Vivo Evaluation of PEP Monomer and Dimer Immunoconjugates**

Because PEP contains a terminal cysteine residue at amino acid 58, we postulated that the synthetic peptide might be forming disulfide-linked dimers during the conjugation procedure. To assess whether dimerization would affect the vasopermeability activity of PEP, we produced both monomer and dimer forms of PEP to use in the conjugation reactions (Fig. 1). The monomer form was created by modifying the sulfhydryl group with N-phenylmaleimide, which eliminated the ability of the monomer to form disulfide bonds at the terminal cysteine. The dimer form was created by incubating the peptide at a high pH to promote the formation of disulfide bonds at the terminal cysteine. The dimer form was created by incubating the peptide at a high pH to promote the formation of disulfide bonds. When the Lym-1 immunocon-

Fig. 2. Biodistribution studies with interleukin 2 (IL-2) and permeability-enhancing peptide immunoconjugates in ME-180 cervical carcinoma-bearing nude mice. Nude mice bearing established ME-180 human cervical carcinoma tumors in the left flank were pretreated by intravenous injection of 30 μg of TNT-1, TNT-1/IL-2, or TNT-1/permeability-enhancing peptide immunoconjugates. After 2 hours, the mice (n = 4 in each group) received intravenous injections of 100 μCi of 125I-labeled TNT-1 tracer in a 0.1-mL inoculum. The mice were killed 72 hours later, and the organs were harvested for biodistribution analysis as described in the “Materials and Methods” section to determine percentage injected dose per gram (A) and tumor-to-organ ratio of tracer uptake (B). Error bars show 95% confidence intervals.
jugate created with the dimer form of PEP was used as a pretreatment in Raji lymphoma-bearing nude mice, biodistribution analysis demonstrated that 5.4% of the tracer antibody was taken up by the tumor, approximately the same uptake as when unmodified PEP was used (i.e., 5.6%). By contrast, the immunoconjugate created with the PEP monomer resulted in tumor tracer antibody uptake of 2.8% (Fig. 3). This result indicated that dimerization of PEP is important in the generation of optimal vasopermeability at the tumor site in these model systems.

Cytokine Activity of PEP and PEP Conjugates

Both unconjugated PEP (monomer and dimer) and TNT-1/PEP were tested for cytokine activity in the HT-2 cell proliferation assay. As shown in Fig. 4, neither unconjugated PEP nor the TNT-1/PEP immunoconjugate was able to promote the proliferation of the IL-2-dependent HT-2 cells over a broad range of concentrations (0.63–40 ng or 0.16–10 U). By contrast, unconjugated IL-2 was highly active in this assay in a dose-dependent manner.

DISCUSSION

In these experiments, a panel of peptide fragments of IL-2 were synthesized and chemically linked to Lym-1, a monoclonal antibody directed against the human HLA-DR10 (42,43) antigen expressed on the surface of human lymphoma cells, or TNT-1, a monoclonal antibody directed against DNA–histone complexes accessible in degenerating cells in solid tumors (31,32,37), and the immunoconjugates were assayed for vasopermeability activity. Physical linkage of the peptide fragments to these tumor-targeting monoclonal antibodies was essential for the peptides to have biologic activity because it presumably lengthened the half-life of the peptides, enabling them to exert their vasopermeability-inducing effect at the tumor site. The results of these studies demonstrate that the vasopermeability activity of IL-2 can be mapped to a peptide fragment (PEP) consisting of a continuous linear sequence of IL-2 (amino acids 22–58). As shown in Fig. 5, this region of IL-2 partially overlaps with the receptor binding site of the molecule. PEP, however, is devoid of cytokine activity as demonstrated by its inability to induce proliferation of the IL-2-dependent T-cell line, HT-2. When administered intravenously as a 2-hour pretreatment, antibody/PEP induced a rapid and reversible vasopermeability of tumor vessels. After linkage to Lym-1, dimers of PEP were more active than monomers, suggesting that dimers are the predominant active species due to the presence of a terminal cysteine at amino acid 58. Moreover, antibody/PEP had the same vasopermeability activity as antibody/IL-2, which was 2.5 to four times that of antibody alone.
With regard to the cause of this vasopermeability, early studies of IL-2 toxicity reported that the cytokine itself had a direct cytotoxic effect on endothelial cell architecture. This conclusion was based on electron microscopy studies performed in mice at the time of autopsy, which found that, shortly after the administration of IL-2, microfenestrations appeared in the endothelial cell tight junctions because of the rounding up of endothelial cells. This reversible condition enables the efflux of fluid but not cells into the extravascular space. It has also been suggested that IL-2 might have an indirect role in the induction of vasopermeability, in which IL-2 promotes vasopermeability through its generation of lymphokine-activated killer cells and/or secondary cytokines that in turn damage the endothelial cells. Secondary cytokines implicated in this indirect effect include tumor necrosis factor-α, IL-1, and interferon gamma, which may increase the expression of the inducible form of nitric oxide synthase (iNOS). This increase in iNOS expression would result in a corresponding increase in nitric oxide (NO), a free radical second messenger that mediates a variety of physiologic functions but is toxic to endothelial cells. In addition to directly damaging the vasculature, NO causes systemic hypotension via its intrinsic vasodilator activity, which can lead to the clinical outcomes of capillary leak syndrome characterized by pulmonary hypertension and edema.

Support for the hypothesis that IL-2 acts indirectly through a secondary cytokine-mediated effect on iNOS comes from the finding that capillary leak syndrome in experimental animals can be prevented or reduced by iNOS inhibitors such as N\textsubscript{G}-methyl-L-arginine (NMA) and N\textsubscript{G}-nitro-L-arginine methyl ester. Previous results from our laboratory further support the role of NO as a mediator of the induction of vasopermeability by IL-2. That is, we found that induction of enhanced permeability of tumor microvasculature by IL-2 delivered specifically to tumor tissue, either by chemical conjugation to a tumor-targeting antibody (Lym-1, TNT-1) or via a genetically engineered antibody/IL-2 fusion protein, was blocked by the simultaneous administration of NMA. Although we did not examine the effect of NMA on the ability of the Lym-1/PEP immunoconjugate to enhance vasopermeability in the current study, it is likely that Lym-1/PEP acts through NO in the same manner as Lym-1/IL-2.

The activity profile of PEP therefore provides evidence that IL-2 vasopermeability is not necessarily the downstream effect of the cytokine activity of IL-2 or binding to the IL-2 receptor. The association of IL-2 vasopermeability activity with NO production may explain why IL-2 and PEP appear to act at a distance to produce rapid and transient vasopermeability in targeted tissues, such as tumors—that is, direct targeting to endothelial cells is not required. As summarized in Table 2, previous data from our laboratory have shown that targeting IL-2 to different components of the tumor also produces permeability in tumor vessels. That is, antibodies directed against the tumor cell surface (Lym-1, B72.3, NR-LU-10), against necrotic areas of tumors (chTNT-1, chTNT-3), or against tumor vascular components such as the basement membrane (TV-1), to which IL-2 or PEP was attached by chemical conjugation.
gation or genetic engineering, all induced marked vasopermeability in the tumor (55,56). By contrast, an IL-2 immunonjugate with a nontargeting antibody, such as chCLL-1 (11), which is directed against human B-cell lymphoma, did not induce enhanced vasopermeability when administered in nude mice bearing solid tumors. These results suggest that, for NO to be released, IL-2 or PEP must be targeted to the tumor. When threshold levels of IL-2 or PEP are reached, NO is presumably released from an unknown cellular source and quickly permeates through the tumor, causing transient vasopermeability and greater uptake of tracer in tumor.

Our results have several important implications. First, the finding that the vasopermeability activity of IL-2 is restricted to a defined portion of the molecule is an important step toward discovering the mechanism of this activity. In this context, it is interesting to note that Baluna et al. (57) have found that amino acids 15–22 of IL-2 form a structural motif, also found in bacterial toxins, that may be involved in the induction of capillary leak syndrome. This area of IL-2 is outside the vasopermeability activity domain that we have defined (i.e., amino acids 22–58). The apparent discrepancy may suggest that vasopermeability is only one contributing factor in the induction of capillary leak syndrome and that other regions of the molecule may be needed to produce the constellation of symptoms associated with this syndrome. Indeed, Mustafa et al. (58) recently proposed that CD44 (hyaluronic acid) may be responsible for IL-2 binding to endothelium. It is likely that the binding site for CD44 on the IL-2 molecule is outside the PEP region. Second, on a more practical note, the availability of PEP enables the generation of a vasopermeability-enhancing agent with a single mechanism of action that has the potential to be used to improve the uptake of macromolecules in tumors and other targeted tissues, as originally described by our laboratory (11,24,25,36,56). Finally, the mapping of vasopermeability to an area of IL-2 that only partially overlaps the known receptor binding area (Fig. 5) gives hope that it may be possible to alter the genetic sequence of IL-2 to produce an analog that retains cytokine functionality but lacks the toxic effects of vasopermeability induction.

Table 2. Effects of pretreatment with chemical and genetically engineered immunoconjugates of interleukin 2 (IL-2) and permeability-enhancing peptide (PEP) on tumor uptake of tracer antibody*  

<table>
<thead>
<tr>
<th>Immunoconjugate</th>
<th>Tracer</th>
<th>Tumor model</th>
<th>Target antigen (reference)</th>
<th>Tumor uptake: % ID/g (95% confidence interval)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
| chCLL-1/IL-2    | chCLL | ME-

- References


