

Use of Uteroglobulin for the Engineering of Polyvalent, Polyspecific Fusion Proteins*

Received for publication, May 27, 2009, and in revised form, July 14, 2009. Published, JBC Papers in Press, July 24, 2009, DOI 10.1074/jbc.M109.025924

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We report a novel strategy to engineer and express stable and soluble human recombinant polyvalent/polyspecific fusion proteins. The procedure is based on the use of a central skeleton of uteroglobulin, a small and very soluble covalently linked homodimeric protein that is very resistant to proteolytic enzymes and to pH variations. Using a human recombinant antibody (scFv) specific for the angiogenesis marker domain B of fibronectin, interleukin 2, and an scFv able to neutralize tumor necrosis factor- α , we expressed various biologically active uteroglobulin fusion proteins. The results demonstrate the possibility to generate monospecific divalent and tetravalent antibodies, immunocytokines, and dual specificity tetravalent antibodies. Furthermore, compared with similar fusion proteins in which uteroglobulin was not used, the use of uteroglobulin improved properties of solubility and stability. Indeed, in the reported cases it was possible to vacuum dry and reconstitute the proteins without any aggregation or loss in protein and biological activity.

The generation of recombinant polyvalent and/or polyspecific fusion proteins for use as components of novel drugs is still hindered by factors that limit their production, storage, and use, chief of which are issues related to instability and/or inadequate solubility. Here we describe a novel approach based on the use of uteroglobulin (UG)³ as a skeleton for the generation of polyvalent/polyspecific recombinant proteins. Human UG is a small (15.8 kDa) globular, nonglycosylated, and homodimeric secreted protein that was discovered independently by two groups in the 1960s in rabbit uterus (1, 2), and it is the first

member of a new superfamily of proteins, the so-called Secretoglobins (Scgb) (3). UG is present in the blood at a concentration of about 15 μ g/ml and is found in urine and in other body fluids. The UG monomer is composed of about 70 amino acids, depending on the species, and is organized in a four α -helix secondary structure; the two subunits are joined in an anti-parallel fashion by disulfide bridges established between two highly conserved cysteine residues in amino- and carboxyl-terminal positions (4) (see Fig. 1). The exact functions of UG are not yet clear, but the protein has been reported to have anti-inflammatory properties due to its ability to inhibit the soluble phospholipase A₂. Moreover, UG contains a central hydrophobic cavity able to accommodate hydrophobic molecules such as progesterone, retinol, and prostaglandin D₂. Theoretically, this cavity could be loaded with different types of therapeutic hydrophobic substances and delivered to targets (for exhaustive reviews on UG, see Refs. 5, 6 and references therein).

The high solubility and stability of UG to pH and temperature variations, its resistance to proteases, and its homodimeric structure prompted us to consider the protein as a candidate linker for the generation of polyvalent and polyspecific recombinant proteins. We demonstrate here that the use of UG as a linker could provide a general method for the generation of covalently linked bivalent and tetravalent antibodies, either monospecific or bispecific, as well as of different kinds of fusion proteins, which, compared with similar fusion proteins without UG, possess generally enhanced properties of solubility and stability, factors that expedite their storage and clinical use.

We describe the use of UG for the production of a bivalent and tetravalent format of L19, an scFv specific for the angiogenesis-associated extra domain B (ED-B) of fibronectin (FN) (7), of an immunocytokine composed of IL2 and L19, and of a tetravalent dual specificity antibody composed of L19 and the scFv D2E7, a human antibody able to neutralize TNF- α activity (8). We report and discuss the characterization, properties, and the biological activity, both *in vitro* and *in vivo*, of these molecules.

EXPERIMENTAL PROCEDURES

Human and Mouse UG cDNAs—Human UG cDNA was obtained by reverse transcription-PCR from normal human lung RNA using the Titan One-Step reverse transcription-PCR system (Roche Diagnostics), with primers TI-36 (see Table 1 for primers sequences) and TI-18, containing the EcoRI and NotI restriction sites, respectively. The resulting product was digested EcoRI/NotI and was ligated into EcoRI/NotI-digested

* This work was supported by the Comitato Interministeriale Programmazione Economica, Rome, Regione Liguria, Genoa (to L. Z.), European Union Grants FP6 LSHC-CT-2003-503233-STROMA and FP6 LSHC-CT-2006-037489-ImmunoPDT (to L. Z.), Istituto Superiore di Sanità, Rome, Grant ISS2006, rare diseases (to B. C. and L. B.), Italian Ministry of Health, Rome, Grant RF-IST-2006-384590 (to L. B.), and Alleanza Contro il Cancro Grant ACC2007 (to L. B.).

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³ The abbreviations used are: UG, uteroglobulin; FN, fibronectin; B-FN, extra domain B containing fibronectin; scFv, single chain variable fragment; SIP, small immunoprotein; CHO, Chinese hamster ovary; TNF- α , tumor necrosis factor- α ; hTNF- α , human TNF- α ; IL, interleukin; PBS, phosphate-buffered saline; ELISA, enzyme-linked immunosorbent assay.

TABLE 1

Sequences of the primers used	
TI-11 forward	5'-TCAAGCTTGTGCGACCATGGGCTGGAGCCTGATC-3'
TI-17 reverse	5'-CGGCGGCCGCTCAGAATCTTAAATCTTGCTTACACAGAGG-3'
TI-18 reverse	5'-CGGCGGCCGCTCAGAATCTTAAATCTTGCTTACACAGAGG-3'
TI-21 forward	5'-CCAAGCTTGTGCGACCATGGGCTGGAGCCTGATCC-3'
TI-22 reverse	5'-GAGGATCCGCCGCTGGACGATGAGCCGGAAGAGC-3'
TI-23 forward	5'-CTGGATCCGAGATCTGCCCGAGCTTTCAGCGTGCATC-3'
TI-24 forward	5'-GCGGATCCCTCTTCGGACATCTGCCAGGATTTCTTC-3'
TI-36 forward	5'-CGGAATTCAGAGATCTGCCCGAGCTTTCAGCGTGCATC-3'
TI-53 reverse	5'-GAGCGGCCGCTCCGAATCTTAA-3'
TI-58 forward	5'-TAGCGGCCGCTCTTCCTCATCG-3'
TI-59 reverse	5'-GGTCTAGAATCAAGTCAGTGTG-3'
TI-65 forward	5'-CTCGGGCCGAGGTGCAGCTGTTGGAG-3'
TI-66 reverse	5'-CTCTCTAGAACTAGAATCTTTGATTCCAC-3'
TI-68 forward	5'-CTCGGGCCGCTCTTCTCATCGGGTAGTAGCTTCCGGCTCATCGTCCAGCGGCGAGGTGCAGCTGTTGGAG-3'
TI-73 forward	5'-CTCGGGCCGCTCTTCTCATCGGGTAGTAGCTTCCGGCTCATCGTCCAGCGGCGAGGTGCAGCTGTTGGAG-3'
TI-74 reverse	5'-CTCTCTAGATCATCATTTGATTCCACCTTGGT-3'
TI-75 forward	5'-CTCGAGGTGCAGCTGGTGGAGTCT-3'
TI-79 reverse	5'-CTCGGGCCGCAATACACAGTGGAGCTTTGGGCTATTTTTT-3'

pProEX-1 vector (Invitrogen). Mouse UG cDNA sequence, provided by GenScript Corp. (Piscataway, NJ), was inserted into the vector pProEX-1.

L19-hUG and L19-mUG cDNAs—From the construct L19-TNF- α , as described previously (9), we amplified by PCR the signal peptide, the portions encoding for L19, and the 15 amino acid linker using the primers TI-21 and TI-22 (9, 10). The resulting product was digested HindIII/BamHI and was ligated into the expression vector pcDNA3.1 (Invitrogen). Human and mouse UGs were amplified by PCR from the clones described above using the primers TI-23 and TI-18 for human UG and the primers TI-24 and TI-17 for mouse UG. The resulting products of PCR were digested BamHI/NotI and were inserted into BamHI/NotI-digested pcDNA3.1/L19-linker, to form pcDNA3.1/L19-hUG and pcDNA3.1/L19-mUG.

L19-mUG-IL2 cDNA—From the construct pcDNA3.1/L19-mUG (see above), we amplified by PCR the sequence of the signal peptide, L19, the linker, and mUG minus the stop codon using the primers TI-11 and TI-53. The obtained sequence was inserted into HindIII/NotI digested vector pcDNA3.1. We obtained the sequence encoding for the linker and IL2 by PCR from the construct pcDNA3.1/L19-IL2 as described in Carnemolla *et al.* (11) using the primers TI-58 and TI-59. The cDNA fragment was inserted into NotI/XbaI-digested pcDNA3.1/L19-mUG to generate pcDNA3.1/L19-mUG-IL2.

L19-hUG-L19 and L19-mUG-L19 cDNAs—From the construct pcDNA3.1/L19-hUG described above, the cDNA containing the sequences coding for the signal peptide, L19, the linker, and hUG minus the stop codon was obtained by PCR using the primers TI-11 and TI-79. To generate the cDNA sequence linker L19 to append at the 3' site of the construct described above, we amplified the L19 sequence by PCR from pcDNA3.1/L19-IL2 (11) using the primers TI-65 and TI-66. The resulting sequence was then used as a template for another PCR with the primers TI-68, including the complete linker sequence, and TI-66. Finally, the sequences composed of HindIII/NotI-digested signal peptide-L19-linker-hUG and of NotI/XbaI-digested linker L19, respectively, were ligated and inserted into the HindIII/XbaI digested pcDNA3.1 to form pcDNA3.1/L19-hUG-L19.

For the generation of the construct pcDNA3.1/L19-mUG-L19, the construct pcDNA3.1/L19-mUG-IL2 described above was digested with NotI/XbaI to remove the linker and IL2 sequences. The sequence encoding for linker L19, as described above, was digested with NotI/XbaI and ligated together with NotI/XbaI-digested pcDNA3.1/L19-mUG to generate pcDNA3.1/L19-mUG-L19.

L19-mUG-D2E7 and L19-hUG-D2E7—For the sequence linker D2E7, we first amplified PCR D2E7 from pcDNA3.1/D2E7-mUG using the primers TI-75 and TI-74. Subsequently, the obtained sequence was amplified with the primers TI-73, containing the complete linker sequence, and TI-74. The resulting sequence, encoding for linker-D2E7, was inserted into NotI/XbaI-digested pcDNA3.1/L19-mUG (see above) to form pcDNA3.1/L19-mUG-D2E7.

For the generation of L19-hUG-D2E7, the construct pcDNA3.1/L19-mUG-D2E7 was digested with BamHI/NotI to remove the mUG sequence. Human UG was then inserted into the open vector to generate pcDNA3.1/L19-hUG-D2E7.

Reagents—All the cDNA constructs were used to transform DH5 α -competent bacteria cells, and clones were selected in Luria Bertani broth (LB) with 100 μ g/ml ampicillin. Clones were screened by PCR. The plasmid DNAs were purified from positive clones using the PureLink HiPure Plasmid Filter Maxiprep kit (Invitrogen), and the DNA sequences were confirmed by sequencing the DNA on both strands.

All PCRs were performed with high fidelity PWO DNA polymerase (Roche Diagnostics) according to the manufacturer's instructions. All restriction enzymes were from Roche Diagnostics. All the PCR products and digested cDNA fragments were purified with the High Pure PCR Purification kit (Roche Diagnostics). The digested vectors were purified by gel-agarose and gel extraction with the QIAquick gel extraction kit (Qiagen, Hilden, Germany).

The purified constructs, with the exception of pProEX/hUG and pProEX/mUG, were used to transfect CHO cells (American Tissue Type Culture Collection, Manassas, VA) using Lipofectamine 2000 CD reagent (Invitrogen) according to the manufacturer's instructions. Transfectomas were grown in RPMI 1640 (Euroclone, Pavia, Italy) supplemented with 10% fetal bovine serum (Biocrom AG, Berlin, Germany) and 4 mM

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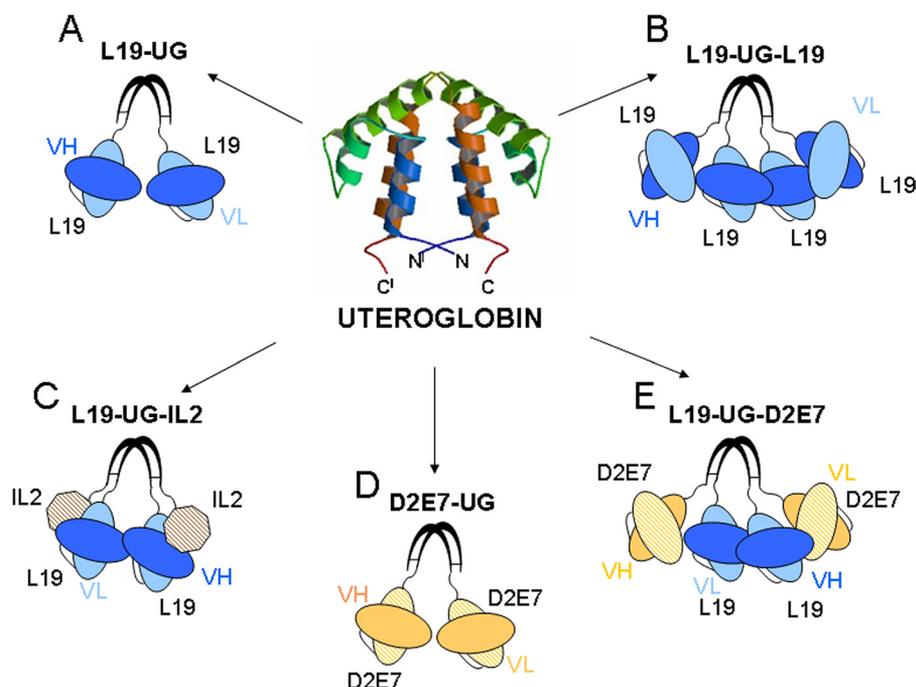


FIGURE 1. Central part of the figure depicts the ribbon structure of the oxidized homodimer of UG (adapted with permission from Ref. 4). A–E show the schemes of the various fusion proteins produced using UG as a central core. L19 is an scFv specific for the angiogenesis-associated FN isoform, and D2E7 is an scFv able to neutralize TNF- α .

L-glutamine (Invitrogen), and selected using 500 μ g/ml geneticin (G418, Calbiochem).

The supernatants of the G418-resistant clones were screened for the production of the fusion proteins by ELISA. The recombinant peptide composed of the type III homology repeat 7B89 (12) was used as antigen for fusion proteins containing L19 antibody and recombinant human TNF- α (PeproTech, Rocky Hill, NJ) for fusion proteins containing D2E7. Rabbit polyclonal anti-mouse UG or anti-human UG antibodies (produced in our laboratory) were used as secondary antibodies, and a peroxidase-conjugated anti-rabbit immunoglobulin G (IgG) polyclonal antibody (Pierce) was used as tertiary antibody.

Fusion proteins were immunopurified from the conditioned media of the cells on ED-B (12) or recombinant hTNF- α (PeproTech, Rocky Hill, NJ) conjugated to Sepharose 4B (Amersham Biosciences). Immunopurified proteins were analyzed in native conditions by fast protein liquid chromatography on a Superdex200 column (Amersham Biosciences) and by SDS-PAGE (4–12% gradient) under reducing and nonreducing conditions.

Radioiodination and Biodistribution Experiments Using L19-UG, L19-UG-L19, and L19-SIP—Proteins were radioiodinated as described previously (13). Purified fusion proteins were radiolabeled with 125 I using the IODO-GEN method (Pierce). After labeling, the immunoreactivity of the fusion proteins was more than 90%. 129/SvHsd mice (Harlan Italy, Udine, Italy) with subcutaneously implanted F9 murine teratocarcinomas were injected intravenously with about 5 μ g (4 μ Ci; 0.148 MBq) of protein in 100 μ l of saline solution. Three animals were used for each time point. Mice were killed at 4, 24, and 48 h after injection. The organs were weighed, and the radioactivity was counted using a gamma counter. Target-

ing results are expressed as a percent of the injected dose per g of tissue (% ID/g).

In Vivo Treatment of Tumor-bearing Mice with L19-UG-IL2 and Assay of TNF- α Neutralizing Activity of D2E7-containing Fusion Proteins—The *in vitro* assay of IL2 biological activity was carried out by testing the ability of fusion proteins to induce T-cell proliferation using CTLL cells (ATCC) as described previously (14).

Treatment with purified L19-UG-IL2 fusion protein was performed as described by Carnemolla *et al.* (11) in groups of six 129/SvHsd mice (Harlan Italy, Udine, Italy), each injected subcutaneously with 3×10^6 F9 cells. Six days after grafting F9 teratocarcinomas into syngeneic mice, tumors reached a volume of nearly 0.3 cm 3 , and three groups of four animals each were then treated for 6 days with a daily intravenous injection in the tail vein of 250,000

units (equivalent) of IL2 as L19-IL2 or L19-UG-IL2. Controls received saline alone. The tumor volume was determined with the following formula: $(d)^2 \times D \times 0.52$, where d and D are the short and long dimensions (centimeters) of the tumor, respectively, measured with calipers (15). Housing, treatment, and killing of animals followed national legislative provisions (Italian law no. 116 of 27 January, 1992) for the protection of animals used for scientific purposes.

The ability of the D2E7-containing fusion proteins to neutralize hTNF- α activity was assessed using the cytotoxicity test on LM fibroblasts (ATCC) as described previously (9, 16). LM cells were treated with 1 μ M recombinant hTNF- α (PeproTech, Rocky Hill, NJ) in the presence of different concentrations of L19-mUG-D2E7 or D2E7-mUG. The data are expressed as percentage of inhibition of TNF- α cytotoxicity.

ELISA Procedures for Studying the Reactions of L19 and D2E7 Moieties within L19-UG-D2E7 with the Respective Antigens—L19-hUG-D2E7 was tested in ELISA against recombinant FN fragments composed of the type III homology repeats 7B89. L19-UG was used as control. ELISA plate wells were coated with 10 μ g/ml 7B89. After washing with PBS, wells were incubated with L19-UG-D2E7 or L19-UG at concentrations ranging from 0.03 to 20 nM, in 2% bovine serum albumin in PBS. A rabbit polyclonal anti-UG antibody (produced in our laboratory) was used as secondary antibody, and a peroxidase-conjugated anti-rabbit IgG polyclonal antibody (Pierce) was used as tertiary antibody. Similarly, L19-UG-D2E7 was tested against human TNF- α (PeproTech), using D2E7-UG as control (Fig. 4, D and E).

The ability of L19 and D2E7 moieties within the L19-UG-D2E7 molecule to simultaneously bind the respective antigens in solution was assessed by incubating ELISA plate wells coated

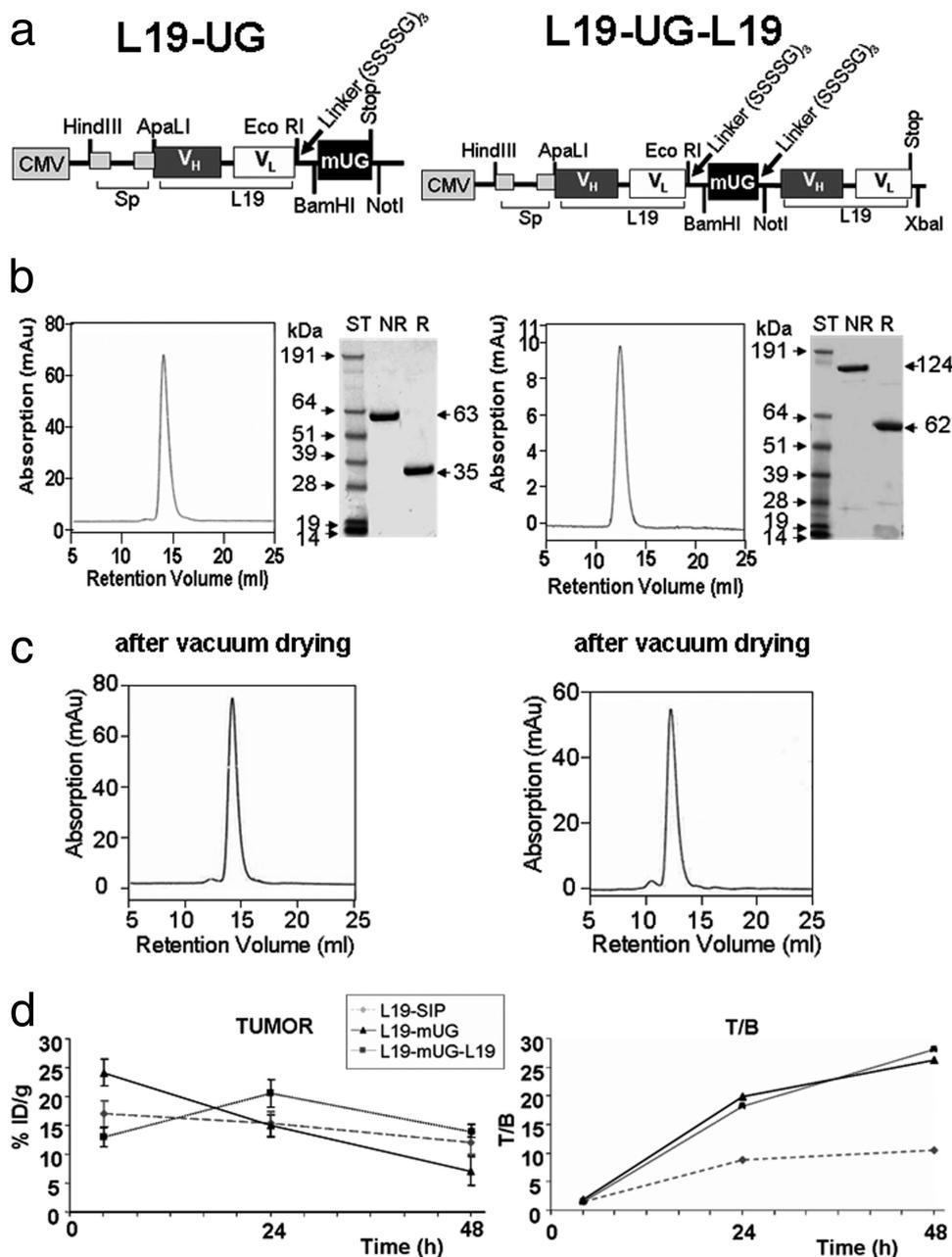


FIGURE 2. *a*, scheme of the cDNA constructs of L19-UG (*left*) and L19-UG-L19 (*right*). *Sp*, signal peptide sequence. *b*, size exclusion chromatography profiles (Superdex 200) and SDS-PAGE analysis of the purified fusion proteins L19-UG (*left*) and L19-UG-L19 (*right*). Nonreducing (NR) and reducing (R) conditions, respectively. *ST*, molecular mass standard. *c*, size exclusion chromatography profiles of L19-UG and L19-UG-L19 reconstituted in distilled water after vacuum drying. *d*, comparative biodistribution experiments in F9 teratocarcinoma-bearing mice of three radioiodinated L19 formats: L19-UG, L19-UG-L19, and L19-SIP. The % ID/g in the tumor at the indicated times after intravenous injection is shown on the *left* (mean \pm S.D.). To the *right* the tumor to blood ratio of the % ID/g at different times after injection of the radioiodinated proteins are plotted. *V_L*, variable light region of immunoglobulin; *CMV*, cytomegalovirus; *mAu*, milli-absorbance units.

with 5 μ g/ml human TNF- α (PeproTech) with 0.8 nM L19-UG-D2E7 in 2% bovine serum albumin in PBS in the presence of 100 μ M of ED-B. Bound L19-UG-D2E7 was then detected using the secondary and tertiary antibodies as described above. As a control the same experiment was performed using 7B89 as immobilized antigen (Fig. 5A).

The ability of L19 and D2E7 moieties within the L19-UG-D2E7 molecule to simultaneously bind the respective antigens in solid phase was evaluated using human TNF- α (PeproTech)

as antigen and 10 μ g/ml L19-UG-D2E7 as primary antibody. Excess protein was washed, and 10 μ g/ml of 7B89 was added. After removing excess 7B89, the bound 7B89 was detected using the anti fibronectin type III repeat 9 monoclonal antibody, HFN 7.1 (ATCC). A peroxidase-conjugated anti-mouse IgG polyclonal antibody (Pierce) was used as tertiary antibody (Fig. 5, *B* and *C*). In all ELISAs the peroxidase activity was visualized using ABTS (Roche Diagnostics); the plates were then read at 405 nm using a SPECTRA-MR (Dynex Technologies, Chantilly, VA).

RESULTS

Fig. 1 shows the ribbon representation of the molecular structure of oxidized UG (4). The UG monomer structure is composed of four α -helices. The two monomers of human UG are held together in anti-parallel fashion by two disulfide bonds between Cys-3 and Cys-69' and the other between Cys-3' and Cys-69. Fig. 1, *A–E*, depicts the hypothetical domain structures of the various fusion proteins containing UG that we describe here as follows: dimeric (Fig. 1A) and tetrameric (Fig. 1B) formats of the scFv L19 (specific for the ED-B of FN, a marker of angiogenesis); an immunocytokine composed of L19 and IL2 (Fig. 1C); the dimeric format of the scFv D2E7 (a human scFv able to neutralize TNF- α activity) (Fig. 1D); and a trivalent dual specificity antibody composed of L19 and D2E7 (Fig. 1E).

L19-UG and L19-UG-L19—To produce the divalent L19, we prepared a cDNA construct composed of the scFv L19 cDNA connected at the 3' end to the 5' end of the UG cDNA, and to produce the tetra-valent L19 format, we appended L19 cDNA at both 3' and 5' ends of the UG cDNA. The resulting L19-UG and L19-UG-L19 constructs (Fig. 2*a*) were then cloned into the vector pcDNA3.1 and used to transfect CHO cells grown in ProCHO5 animal protein-free media (Lonza, Verviers, Belgium) to produce 5–10 mg/liter recombinant protein that can be efficiently purified either on ED-B (the antigen of L19) or protein-A affinity chromatography because the variable heavy region of immunoglobulin chain of L19 belongs to the subgroup III and thus contains protein A-binding sites (7, 17).

UG in the Preparation of Polyspecific Recombinant Proteins

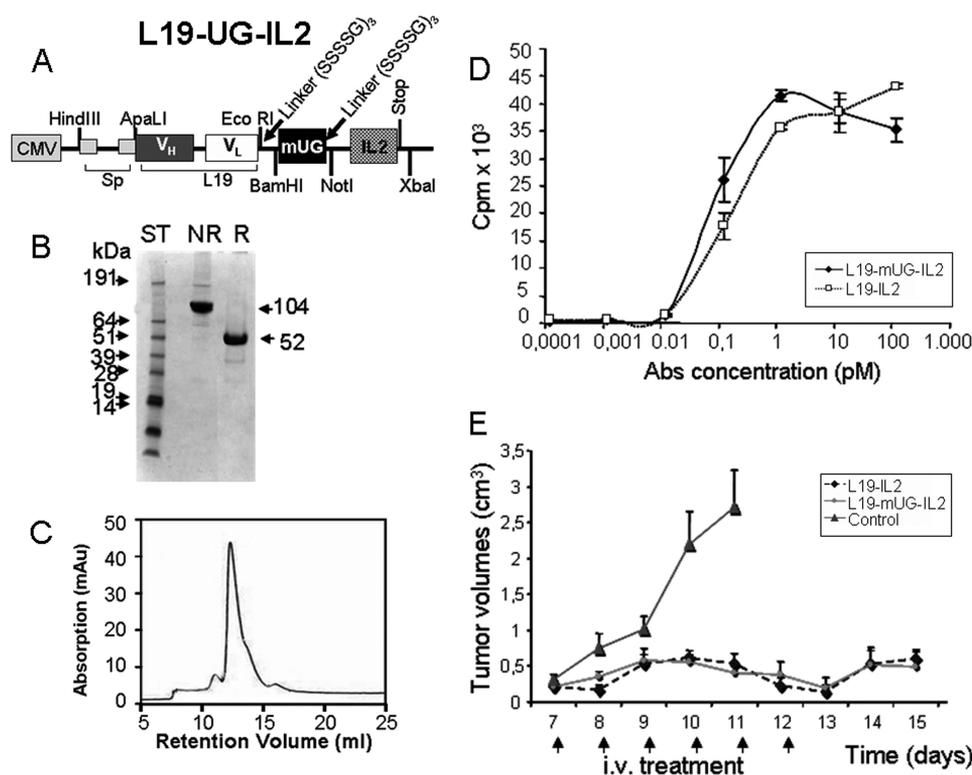


FIGURE 3. *A*, scheme of the cDNA construct of L19-UG-IL2. *Sp*, signal peptide sequence. *B*, SDS-PAGE analysis (nonreducing (NR) and reducing conditions, respectively); *C*, size exclusion chromatography profile (Superdex 200) of purified L19-UG-IL2. *D*, proliferation assay on CTLL cells using [3 H]thymidine. Equimolar amounts of L19-IL2 and L19-UG-IL2 induced identical thymidine incorporation into cells, indicating that the two proteins share identical IL2 activity. *Abs*, antibodies. *E*, tumor growth inhibition of equimolar amounts of L19-IL2 and L19-UG-IL2 or only saline. Each group consisted of four mice. Therapy started 7 days after tumor implantation. Arrows indicate the days of treatment. Tumor size was determined as reported under "Experimental Procedures." S.E. are indicated. *VL*, variable light region of immunoglobulin; *CMV*, cytomegalovirus; *mAu*, milli-absorbance units.

In SDS-PAGE both purified proteins migrate as homodimers in nonreducing conditions and as monomers in reducing conditions, showing apparent sizes of about 63 and 35 kDa, respectively, for the divalent format and of 124 and 62 kDa, respectively, for the tetravalent format. The apparent size of the nonreduced divalent format was lower compared with the expected size of 70 kDa, very likely due to the compact conformation of the nonreduced molecule. In nonreducing conditions both molecules were more than 95% covalently linked dimers (Fig. 2*b*). The size exclusion chromatography (SEC) profiles of both fusion proteins showed a single peak with a retention volume corresponding to the molecular mass of the homodimers and the absence of aggregates (Fig. 2*b*). Both proteins were soluble in PBS at concentrations over 2 mg/ml, and after vacuum drying, these proteins could be reconstituted without any loss and without the formation of aggregates (Fig. 2*c*). These fusion proteins were generated using both mouse and human UG, and all the proteins presented identical properties. Our group has previously reported on a different covalently linked L19 homodimer obtained using the domain 4 of the constant heavy region of human IgE secretory isoform (small immune protein (SIP)) (13). This protein radiolabeled with ^{131}I is now extensively used in phase I/II radioimmunotherapy trials (18–20). However, L19-SIP, as well as the scFv *per se*, presents a much lower solubility than L19-UG fusion proteins, and it cannot be reconstituted after vacuum drying

without aggregation and precipitation. To obtain a stable solution of SIP, it is necessary to keep it at a concentration not exceeding 0.5 mg per ml and at a temperature of -80°C in the presence of stabilizers such as sucrose or Tween to avoid the formation of precipitates during thawing. By contrast, the UG constructs can be kept in solution in PBS at either -20 or -80°C and thawed without the formation of any aggregates. These properties, together with the possibility to keep the molecules in the dry state, are of noteworthy importance for the storage of agents to be used in therapies in hospitals. We compared the tumor-targeting performance of the three radioiodinated L19 homodimers (L19-SIP, L19-UG, and L19-UG-L19) (Fig. 2*d*) in 129/SvHsd mice bearing the syngeneic F9 teratocarcinoma. As reported previously, the vasculature of the teratocarcinoma F9 presents the accumulation of B-FN (9). Fig. 2*d* (left panel) shows the percentage of injected dose per g of tissue (% ID/g) in the tumor at different times after injection of the three radioiodinated L19 formats. Considering the area

under the curves, L19-UG-L19 performed best, although the L19-UG and the L19-SIP shared a similar area. Fig. 2*d* (right panel) shows the ratio of the % ID/g of tumor and of blood for the three L19 formats as follows: in this case the tumor/blood ratio was more than two times higher for the two UG formats than for the SIP format, because of the faster blood clearance profile of the former. The rapid clearance of molecules used for immunoradiotherapy is quite important because it limits the exposure of other organs to radiation.

The ratios of the % ID/g in the tumor *versus* other organs were in all cases, at 48 h from injection of the radioiodinated proteins, greater than 10 (data not shown). The biodistribution of these L19 formats in other experimental tumor models was also studied, and in all cases the UG formats performed better than the SIP format.⁴

L19-UG-IL2—To demonstrate that UG can also be used to generate active immunocytokines in the format of stable covalently linked homodimers, we expressed the immunocytokine L19-UG-IL2. The choice of this immunocytokine was prompted by the fact that we had previously produced L19-IL2 without UG (11); therefore, this allowed us the opportunity to compare the properties and the biological activity of the two molecules and to validate the use of UG for the generation of

⁴ L. Zardi, manuscript in preparation.

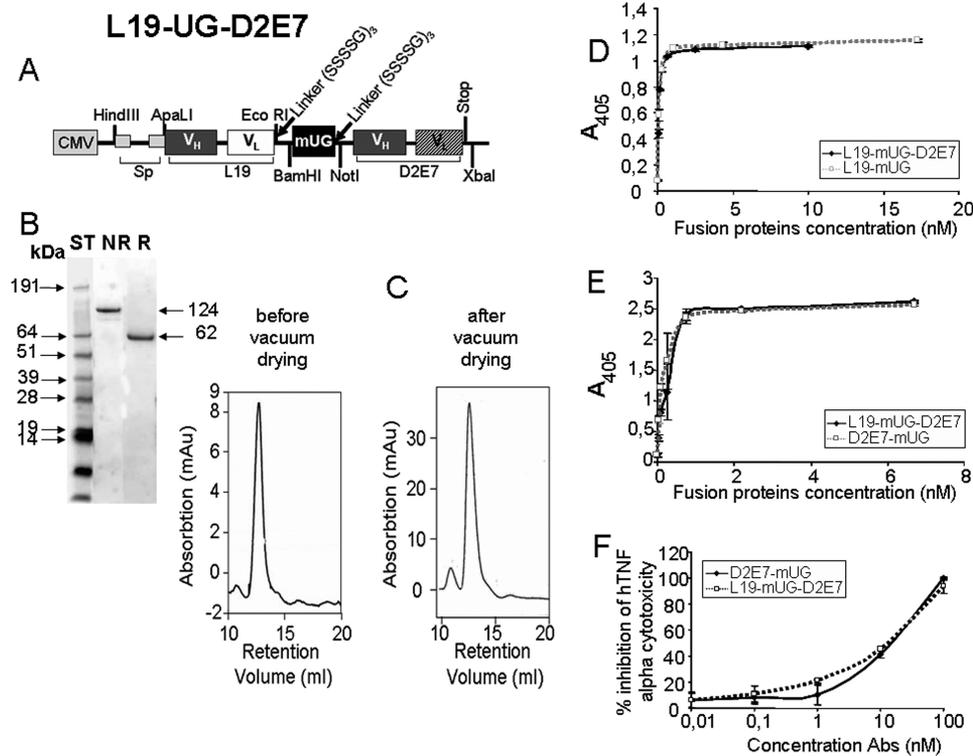


FIGURE 4. A, scheme of the cDNA construct of L19-UG-D2E7. Sp, signal peptide sequence. CMV, cytomegalovirus. B, SDS-PAGE analysis under nonreducing (NR) and reducing (R) conditions; ST, molecular mass standard. C, size exclusion chromatography profile (Superdex 200) of purified L19-UG-D2E7 (left panel); size exclusion chromatography profile (Superdex 200) of purified L19-UG-D2E7 after vacuum drying (right panel). The binding ability to the antibody-specific antigens was studied in ELISA using different concentrations of D. L19-UG-D2E7 on ED-B of FN (using L19-UG as control); E, against TNF- α (using divalent D2E7-UG as control). F, inhibitory activity of hTNF- α cytotoxicity of different concentrations of L19-mUG-D2E7 and D2E7-mUG on LM mouse fibroblasts treated with 1 μ M hTNF- α . The mean \pm S.D. are reported. Abs, antibodies; mAu, milli-absorbance units.

immunocytokines. As is shown in Fig. 3A, the cDNA construct of L19-UG-IL2 was engineered by recombinant DNA technology by ligating the cDNA of the scFv L19 at the 5' end and the cDNA of IL2 at the 3' end of the UG cDNA. After cloning in the vector pcDNA3.1, CHO cells were transfected, and the clones produced roughly 3 mg/liter recombinant protein. In SDS-PAGE the purified L19-UG-IL2 migrated as a homodimer (more than 95%) in nonreducing conditions and as a monomer in reducing conditions at the expected sizes of about 104 and 52 kDa, respectively (Fig. 3B). The SEC profile also showed a main peak with a retention volume corresponding to the apparent molecular mass of the homodimer (Fig. 3C). In this case too, it was possible to vacuum dry and reconstitute the fusion protein without any loss and without the formation of aggregates (data not shown). On the contrary, the fusion protein without UG could not be vacuum-dried and had to be stored at -80°C with stabilizers to prevent the formation of precipitates during thawing; the fusion protein containing UG, on the other hand, could be kept at -80°C in PBS without any stabilizer and thawed without aggregation and/or formation of precipitates.

We compared the *in vitro* biological activity of IL2 by CTLL proliferation assay (14). Equimolar amounts of L19-IL2 and L19-UG-IL2 showed identical IL2 activity (Fig. 3D). We also compared the *in vivo* ability of L19-UG-IL2 and L19-IL2 to inhibit the growth of F9 teratocarcinoma in syngeneic mice. When the animals reached a volume of nearly 0.3 cm^3 , groups

of animals were treated for 6 days with daily intravenous injections of 250,000 units of IL2 as L19-IL2, L19-UG-IL2, or saline alone. The results depicted in Fig. 3E show that L19-UG-IL2 had an anti-tumor activity identical to that previously reported for L19-IL2 (11).

L19-UG-D2E7—We generated a dual specificity tetravalent molecule using the scFv L19 (anti-ED-B) and the scFv D2E7 (inhibiting TNF- α) with UG as a central skeleton. As is shown in Fig. 4A, the cDNA construct of L19-UG-D2E7 was prepared by ligating the cDNA of the scFv L19 and the cDNA of the scFv D2E7 at the 5' and 3' ends, respectively, of the UG cDNA. This construct was inserted in the vector pcDNA3.1, and CHO cells were transfected; the resulting clones produced about 3 mg/liter recombinant protein. As a control, the fusion protein D2E7-mUG was obtained with a similar approach (data not shown). Fig. 4, B–F, shows the characterization of the purified dual specificity tetravalent molecule L19-UG-D2E7. In SDS-PAGE (Fig. 4B), the purified protein migrated as a homodimer (more than 95%) in

nonreducing conditions, showing the expected mass of about 124 kDa, and as a monomer with a mass of 62 kDa in reducing conditions. The SEC profile (Fig. 4C) showed a main peak with a retention volume corresponding to the molecular mass of the homodimer. In this case, too, it was possible to vacuum dry and reconstitute the fusion protein without any loss and without the formation of aggregates (Fig. 4C). The immunoreactivity of L19-UG-D2E7 was tested by ELISA against the two antigens, the ED-B and TNF- α . The results, shown in Fig. 4, D and E, show that L19-UG and L19-UG-D2E7 reacted equally well with the ED-B (Fig. 4D), and that D2E7-UG and L19-UG-D2E7 reacted equally well with TNF- α (Fig. 4E), thereby demonstrating that the two scFvs within the L19-UG-D2E7 molecule do not interfere with each other. The results of an experiment of TNF- α cytotoxicity inhibition on LM cells using L19-UG-D2E7 and D2E7-UG show (Fig. 4F) that the two formats share a similar neutralizing ability, again demonstrating that the two antibodies on the same molecule do not interfere with each other.

We also demonstrated that in L19-UG-D2E7 each antibody can properly function in either solution or solid phase. Addition of an excess amount of ED-B (100 nM) to L19-UG-D2E7 in PBS containing 2% of bovine serum albumin, although abolishing the reactivity with the immobilized ED-B, revealed no interference with the reactivity of the D2E7 moiety with TNF- α (Fig. 5A). To demonstrate that each binding domain could also function independently in solid phase, ELISA wells were coated with

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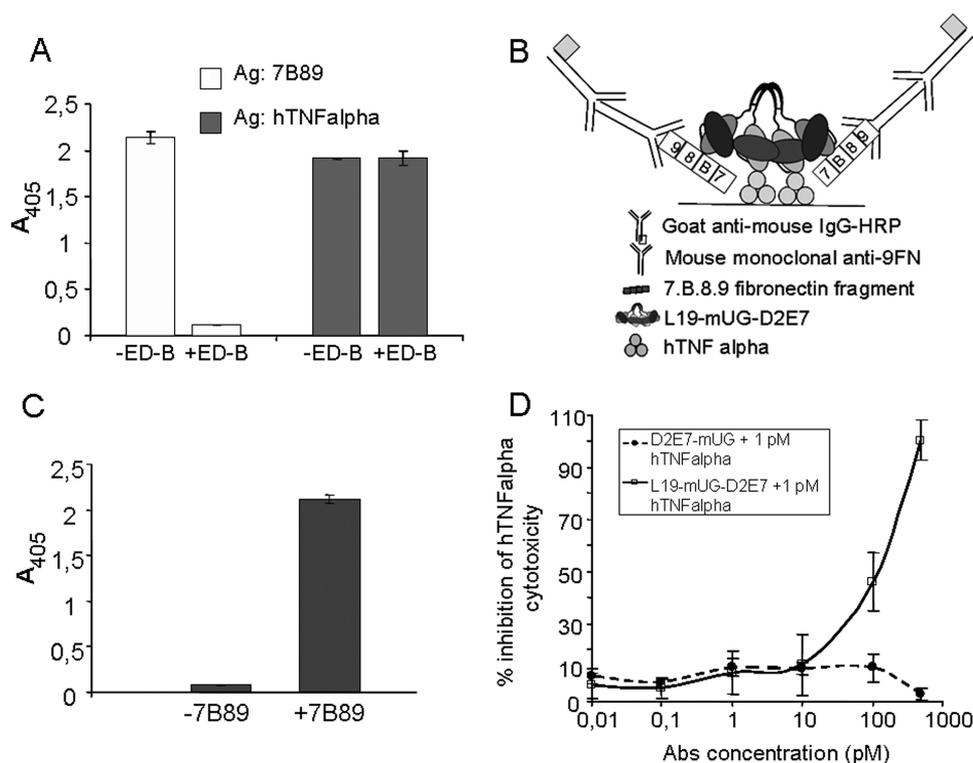


FIGURE 5. A, ELISA using 0.8 nM D2E7-UG-L19 on the ED-B of FN (white bars) or TNF- α (dark gray bars) as antigen (Ag). The reaction was performed with or without 100 μ M ED-B. B, schematic drawing; C, results of ELISA performed using TNF- α as antigen and L19-UG-D2E7 as primary antibody; after removing the excess antibody the FN fragment 7B89 was added and was detected using an antibody specific for the FN repeat 9. D, L19-mUG-D2E7 bound to the ED-B neutralizes TNF- α cytotoxicity (*in situ* neutralization). The cytotoxic activity of 1 pM human TNF- α was evaluated on LM cells using plates pre-coated with 7B89 and preincubated with different concentrations (0.01–500 pM) of L19-mUG-D2E7 (□) or D2E7-mUG (●). After removing unbound antibodies, hTNF- α was inhibited only by L19-mUG-D2E7, because it bound to the ED-B of FN with which the wells were pre-coated. Abs, antibodies.

TNF- α and incubated with L19-UG-D2E7. The excess antibody was washed out, and the FN fragment composed of the type III repeat 7B89 was then added to the well (Fig. 5, B and C). This fragment bound to the L19 moiety and was then detected using a monoclonal antibody specific for the FN repeat 9. The results showed that even when an scFv is bound to the antigen in solid phase, the other is still free to react with its antigen.

Not only was the specific binding ability preserved but the biological activity of the antibodies was as well, as revealed by the inhibition of the TNF- α -mediated cytotoxicity experiments on LM fibroblasts (Fig. 5D). To mimic the targeted delivery of D2E7 in tissues containing B-FN, the inhibitory activity of L19-mUG-D2E7 on hTNF- α cytotoxicity was evaluated on LM cells on culture plates pre-coated with the type III repeat 7B89. D2E7-UG was used as a nontargeted control. After incubation of cells with the fusion proteins and washing out the excess, hTNF- α was added. In this condition only the L19-UG-D2E7 should have been present on the plates, because it binds to the FN fragment with which the plates were coated. The results obtained (Fig. 5D) demonstrate that even when L19 is bound to the extra domain B, the D2E7 moiety is still able to neutralize hTNF- α .

DISCUSSION

The generation of effective proteins, particularly antibody derivatives, is beset by a number of problems, chief of which are

the complex production processes and aggregation and stability issues arising during storage. Despite various attempts to overcome difficulties (21–24), these obstacles remain.

Here we describe a novel and generally applicable approach to generate tetravalent, dual specificity fusion proteins using UG as a central core of the molecules. We found that, compared with formats in which UG was not used, the use of UG as the scaffold enhanced stability and solubility of the fusion proteins. Such improvements in these properties open the possibility to store them at 4 °C in a vacuum-dried state. Indeed, using UG as a central core, we have demonstrated the possibility to produce various immunologically and biologically active, covalently linked fusion proteins, such as divalent and tetravalent scFvs, immunocytokines composed of scFvs and a cytokine, as well as tetravalent dual specificity antibodies.

We generated a divalent and tetravalent format of the human scFv L19, specific for the ED-B containing isoform of FN (25). FN is a large glycoprotein that is present both in plasma and tissues. The ED-B is a 91-amino acid type III homology repeat that is inserted in the FN molecule under tissue-remodeling conditions by preferential alternative splicing of the primary transcript (25). The ED-B is undetectable in tissues from healthy adult individuals, but B-FN is abundant in many aggressive cancers (26–31). The scFv L19 has been shown to efficiently and selectively localize in tumor blood vessels in animal models and in patients with cancer following intravenous injection (13, 15, 18, 32, 33). Thanks to its ability to selectively accumulate in neoplastic tissues, L19 is currently undergoing extensive testing in clinical trials for the radioimmunotherapy of various forms of tumors, being administered as the divalent format of SIP radiolabeled with ¹³¹I (13, 19, 20, 34, 35).

Here we present two novel formats of L19 that seem particularly suitable for immunoradiotherapy and that present considerable advantages compared with the L19 in the scFv and SIP formats previously used. In fact, these novel L19-UG formats performed better than L19-SIP in biodistribution experiments in tumor-bearing mice (Fig. 2d), showing a higher tumor blood ratio of the % ID/g of tissue. In addition, considering that UG is an inhibitor of inflammation and fibrosis (36), two important side effects of radiotherapy, its presence in the radiolabeled protein might help to reduce these unwanted effects. Moreover, both the divalent and tetravalent UG are much more soluble than previously used formats; in fact, in PBS, a concentration of 2 mg/ml without any additive can be achieved without observ-

ing any precipitation or aggregation, qualities that would facilitate the radioiodination process. We conducted vacuum drying experiments using both of the L19-UG formats in PBS and found that it was possible to reconstitute the proteins using distilled water without any loss of protein, precipitation, or formation of aggregates. On the contrary, the SIP did not possess these properties, making its clinical use less easy.

We also generated an immunocytokine composed of the scFv L19 and IL2, and again we demonstrated that this molecule is active in both the antibody and the cytokine moieties. Our group previously prepared a UG-free immunocytokine composed of IL2 and L19 and showed that its fusion with L19 enhanced the therapeutic index of the cytokine (11). This fusion protein is currently being investigated in a multicenter phase II clinical study in Italy and Germany (37). Here we compared the biological activity of L19-UG-IL2 and L19-IL2 both *in vitro* on T-cells and *in vivo* on an experimental animal model and obtained identical results (Fig. 3), thereby demonstrating that UG could be used as a general approach for the generation of biologically active immunocytokines in the form of stable, covalently linked homodimers. In addition, in this case also the inclusion of UG as a scaffold permitted the vacuum drying of the construct, which was not possible with the immunocytokine without UG. Moreover, given its anti-inflammatory properties, UG could be used for the generation of immunocytokines with anti-inflammatory activity, such as IL10. We are currently investigating whether the presence of UG is able to enhance the anti-inflammatory properties of similar cytokines. In fact, it has recently been shown that an immunocytokine made up of the scFv L19 and IL10 selectively accumulates at sites of arthritis in collagen-induced arthritis experimental animal models and that it inhibits disease progression (38). The activity of this anti-inflammatory immunocytokine could be further enhanced using UG, which, again, *per se* possesses anti-inflammatory properties and, in addition, yields a stable covalently linked homodimer.

To further demonstrate the versatility of this procedure, we generated a dual specificity tetraivalent antibody using the scFvs L19 and D2E7. D2E7 is a TNF- α -neutralizing scFv marketed as a complete IgG under the brand name Humira, which has been successfully used for the treatment of rheumatoid arthritis. The use of the dual specificity, tetraivalent L19-UG-D2E7 fusion protein could afford a novel therapeutic approach, namely the *in situ* inhibition of TNF- α , because the presence of L19 would prompt the selective accumulation of the fusion protein at the site of disease sites where D2E7 could explicate its neutralizing activity. This selective delivery would, in turn, diminish the side effects of anti-TNF- α antibody treatment. This approach and the *in situ* inhibition of TNF- α could, of course, be performed using the entire repertoire of proteins able to inhibit TNF- α , including the TNF- α receptor (see Ref. 8 and references therein). Admittedly, all of these hypotheses must be tested *in vivo*, but we have demonstrated the possibility to generate a dual specificity tetraivalent molecule composed of an scFv, L19, which is able to selectively deliver the molecule to the diseased tissue, and by a second scFv that inhibits TNF- α , even when L19 is engaged with its antigen. All of the fusion pro-

teins we have generated displayed optimal solubility properties and the absence of aggregates. We also show that each binding and/or biological active moiety could function independently without interfering with each other in either solution or solid phase.

In conclusion, we describe here a flexible and robust procedure for the generation of fusion proteins suitable for different therapeutic options, namely radioimmunotherapy, photodynamic, anti-inflammatory, and immunocytokine therapies, in a very broad range of angiogenesis-associated pathologies, including cancer and degenerative diseases. In addition, the UG homodimer contains a central hydrophobic cavity with a volume adequate to accommodate hydrophobic molecules such as progesterone, retinol, and prostaglandin D₂. Theoretically, this cavity could be loaded with different kinds of hydrophobic therapeutic substances and delivered to targeted organs or tissues. We are currently investigating such possibilities (6).

Acknowledgments—We thank Sibel Sümer for editorial assistance in the preparation of the manuscript and Thomas Wiley for manuscript revision. We thank Dr. Silvano Ferrini for help in the evaluation of the IL2 activity.

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