Recombinant Anti-Human Melanoma Antibodies Are Versatile Molecules

Dario Neri,* Pier Giorgio Natali,† Heike Petrul,* Patrizia Soldani,‡ Maria Rita Nicotra,§ Roberto Vola, Anna Rivella, Andrew M. Creighton, Paolo Neri‡, and Massimo Mariani**

*Cambridge Center for Protein Engineering, Medical Research Council Center, Hills Road, Cambridge CB2 2QH, U.K.; †Regina Elena Institute, Via delle Messi d'Oro 156, 00158 Roma, Italy; ‡Department of Molecular Biology, University of Siena, Centro Didattico loc. Le Scotte, 53100 Siena, Italy; §Institute of Biomedical Technologies, National Research Council, Roma, Italy; Biochemical Oncology Laboratory, SORIN Biomedica, Saluggia (Vc), Italy; and **Department of Nuclear Medicine, St Bartholomew's Hospital Medical College, West Smithfield, London EC1A 7BE (U.K.)

The low cost, high versatility, and reliable production of bacterially produced recombinant antibody fragments speeds up the development of tumor-targeting agents. High-quality recombinant anti-melanoma antibodies are much sought after in the scientific community. We cloned the murine antibody 225.28S, currently used in radioimmunoimaging of human melanoma lesions, in single-chain Fv configuration (scFv) for soluble expression in bacteria. The recombinant antibody fragment conserved the binding specificity of the parental antibody. In order to arm the scFv(225.28S) with biologically useful effector functions, we developed vectors for soluble expression of scFv(225.28S) in bacteria that allow both covalent and noncovalent chemical antibody modification at positions that do not interfere with antigen binding. An expression vector was developed that appends a cysteine residue at the C-terminal extremity of the recombinant antibody, thus allowing reaction with thiol-specific reagents, including ^{99m}Tc labeling, at a position that does not interfere with antigen binding. The scFv(225.28S) was also successfully expressed with a casein kinase II substrate tag that enables efficient and stable ^{32}P labeling. For noncovalent antibody modification, we developed an expression vector that appends the human calmodulin gene at the C-terminal extremity of scFv(225.28S). The calmodulin domain is poorly immunogenic and can be targeted with chemically modified high-affinity calmodulin ligands. The recombinant anti-human melanoma antibodies described in this article should prove useful "building blocks" for the development of anti-melanoma diagnostic and therapeutic strategies. ***Key words: scFv/recombinant antibodies/human melanoma/antibody functionalization. J Invest Dermatol 107:164–170, 1996***

Analysis of human tumors with monoclonal antibodies (MAbs) has identified a variety of tumor-associated antigens with various degrees of tumor specificity. The restricted tissue distribution of some of these antigens has rekindled interest in the application of radioimaging techniques for diagnostic purposes and possible therapeutic treatments.

MAbs to tumor-associated antigens expressed by melanoma, glioma, and carcinomas of breast, gastrointestinal tract, and ovary have already been successfully used to radioimage (McKearn, 1993) and, in some cases, to treat lesions in patients with the corresponding tumors (e.g., Goldenberg, 1993; Riva *et al*, 1994).

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Reprint requests to: Dario Neri, Cambridge Centre for Protein Engineering, MRC Centre, Hills Road, Cambridge CB2 2QH, U.K.

Abbreviations: CAL, calmodulin; Fab, antibody fragment; HMW-MAA, high-molecular weight melanoma-associated antigen; MAA, melanoma-associated antigen; MAb, monoclonal antibody; protein LG, immunoglobulin binding protein; SAB, second antibody binding; scFv, single-chain Fv.

**Current address: Development Department, I.R.I.S.-BIOCINE, Via Fiorentina, 1, 53100 Siena, Italy.

The high-affinity murine IgG2a monoclonal antibody (MAb) 225.28S (Natali *et al*, 1984) is specific for a high-molecular weight melanoma-associated antigen (HMW-MAA). The antigen shows several biologic features of clinical relevance, namely, its expression by at least 90% of melanoma lesions (Natali *et al*, 1984), the fact that it is less heterogeneous in its expression than other types of MAAs investigated (Natali *et al*, 1983), that it displays a highly-restricted normal tissue distribution (Natali *et al*, 1984), and that it is present only in minute amounts in serum of patients with advanced disease (Giacomini *et al*, 1984).

Previous multicenter studies (Buraggi *et al*, 1985; Siccardi *et al*, 1986, 1987) have shown that the labeled anti-melanoma antibody 225.28S localizes metastatic melanoma lesions in patients. To improve imaging results and to explore possible therapeutic applications, different radionuclide labels and imaging approaches, from direct radiolabeling to multistep techniques based on the avidin-biotin system, have been developed (Paganelli *et al*, 1991). Because 225.28S MAb-based radiopharmaceuticals have been shown to be useful in the management of melanoma patients (Siccardi *et al*, 1987), there is interest in the production of a recombinant molecule, for example, a recombinant single-chain Fv fragment (scFv; Bird *et al*, 1988; Huston *et al*, 1988), which may find therapeutic applications and which the scientific community may use as a

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"targeting unit" for modular melanoma-targeting approaches. We here report the cloning and characterization of the 225.28S antibody in different recombinant formats in terms of performance and functional affinity for its biologic target.

MATERIALS AND METHODS

Antibodies and Cell Lines The generation of MAb 225.28S to a high molecular weight melanoma-associated antigen (Natali *et al*, 1984), MAb 9E10 (Munro and Pelham, 1986), and scFv(D1.3) (McCafferty *et al*, 1990) has been described previously. The anti-225.28S idiotypic antibody MF-11 was a gift from Dr. Soldano Ferrone (New York Medical College, Valhalla, NY). The anti-calmodulin (CAL) human antibody fragment (Fab) DN169/F3 (Neri *et al*, 1995a) was isolated by D.N. as described (Griffiths *et al*, 1994). Purified 225.28S MAb, $F(ab)_2$, and Fab fragments were obtained as described (Mariani *et al*, 1991).

The continuous melanoma cell line COLO-38 was maintained in P.G.N.'s laboratory in RPMI 640 medium supplemented with 10% heat-inactivated fetal calf serum (GIBCO BRL, Gaithersburg, MD) and 2 mM glutamine. The carcinoembryonic antigen-expressing HT29 cell line was obtained from the American Type Culture Collection (Bethesda, MD).

Cloning, Stability, and Functionalization of scFv(225.28S

Cloning of 225.28S in scFv Configuration The rescue of the antibody VH and VL genes followed the strategy described by T. P. Bonnert [PhD Thesis, University of Cambridge (UK), 1994], a modification of the method of Clackson *et al* (1991) in which VH and Vk domains are cloned sequentially in pHEN1-based vectors (Hoogenboom *et al*, 1991). Positive clones were identified by analysis of bacterial supernatants expressing the scFv fragment (Marks *et al*, 1991) for binding to the anti-idiotypic antibody MF-11 (Kusama *et al*, 1989) on a BIAcore biosensor machine (Pharmacia, Piscataway, NJ; Jönsson *et al*, 1991). A clone positive in BIAcore was selected and sequenced according to standard procedures. Its nucleotide sequence and the amino acid sequence have been deposited at the EMBL Sequence Database (accession number X95707). These sequences coincide with those of the parental antibody.

Expression Vectors scFv(225.28S) was subcloned in the Sfi1/*Not*I cloning sites of vectors pDN23, pDN227, and pDN152, yielding clones pDN49, pDN284, and pDN206, respectively. pDN23 is a derivative of pHEN1 (Hoogenboom *et al*, 1991), which does not contain the phage gene III and in which the sequence between the *Not*I and the *Eco*RI sites (*underlined*) has been replaced by the sequence: GCGGCCGCAGGCGGTTCTTCCGGCTCCT-GTGAACAAAAACTCATCTCAGAAGAGGATCTGAATTAATAAGAA-TTC. Cloning of a recombinant antibody in the Sfi1/*Not*I sites of pDN23 results in a C-terminal extremity of the molecule containing a heptapeptide sequence GGSSGSC, followed by the myc tag. We refer to this format as scFv-Cys-myc. pDN227 (Neri *et al*, 1996a) appends the sequence DDD**S**D-DDDYKDDDDK at the C-terminal extremity of the scFv fragment, where the *boldface* serine residue can be phosphorylated by casein kinase II (Marin *et al*, 1986), and the *underlined* sequence corresponds to the FLAG tag (Hopp *et al*, 1988). We refer to this format as scFv-P-FLAG. pDN152 (Neri *et al*, 1995a) appends CAL, a small protein of 148 amino acids, to the C-terminal extremity of the antibody. We refer to this format as scFv-CAL.

Expression and Purification of scFv(225.28S) In a typical protocol, the clone of interest (pDN49/TG1, pDN284/TG1, or pDN206/TG1) was inoculated into 1 liter 2xTY medium containing 0.1% glucose and 100 mg of ampicillin per liter and grown at 37°C until $OD^{600} = 0.8$. Isopropylthiogalactopyranoside was then added to a final concentration of 1 mM, and growth was continued with shaking at 20°C for 16–20 h. The suspension was then centrifuged and the supernatant filtered and concentrated to 1/10 of the original volume using a Filtron Minisette filtration device (Filtron Technology Corp., Northborough, MA), using benzamidine (10 mg per liter) and soybean trypsin inhibitor (10 mg per liter) as protease inhibitors.

The scFv(225.28S)-Cys-myc was purified from this solution by chromatography on either a column prepared with the anti-myc tag antibody 9E10 (Marks *et al*, 1991) or on a column prepared with LG protein (Kihlberg *et al*, 1992; Vola *et al*, 1994) as described. The scFv(225.28S)-P-FLAG was affinity purified on an anti-FLAG resin (M2 resin, Kodak IBI) according to the manufacturer's instructions. The scFv(225.28S)-CAL was purified sequentially by affinity chromatography on *N*-(6-aminohexyl)-5-chloro-1-naphthalenesulfonamide-agarose (Sigma Chemical Co., St. Louis, MO) and by anion exchange chromatography on a Mono-Q column on a Pharmacia FPLC system as described (Neri *et al*, 1995a). In all three cases, the yields were on the order of 1–10 mg per liter of culture. Clones expressing scFv(225.28S) tend to lose expression competence if kept on agar plates and are better stored as glycerol cultures or as plasmids.

Functionalization of scFv(225.28S) Purified scFv(225.28S)-Cys-myc was functionalized with iodoacetamido-LC-biotin (Pierce, Rockford, IL) or with iodoacetamido-fluorescein (Molecular Probes, Eugene, OR) using similar protocols. The antibody (1 mg/ml) was first reduced with 1 mM Tris(2-carboxyethyl)phosphine hydrochloride (Molecular Probes) in PBS (phosphate-buffered saline; 50 mM phosphate buffer, pH 7.4, 100 mM NaCl) for 30 min on ice, and iodoacetamido fluorescein or LC-biotin (2 mM stock in dimethyl formamide) was added to the antibody solution in the volume ratio 1 (reagent solution):10 (antibody solution). The reaction was allowed to proceed for 3 h on ice in the dark and was then quenched with 10 mM dithiothreitol. The residual functionalization reagent was finally removed by gel filtration on PD-10 desalting columns (Pharmacia) equilibrated with PBS.

Fluorescein labeling was checked by sodium dodecyl sulfate-polyacrylamide gel electrophoresis on Homogeneous 20% Phast gels (Pharmacia) and imaged with the cooled CCD-camera gel imaging system LUANA (Neri *et al*, 1996b). Antibody biotinylation was checked by radioimmunoassay after coating plates with the anti-idiotype antibody MF-11 (5 μg per ml) (Kusama *et al*, 1989), and using ^{125}I-labeled streptavidin as detection reagent. ScFv(225.28S)-P-FLAG was phosphorylated with [γ-^{32}P]ATP and casein kinase II as described (Neri *et al*, 1996a).

Serologic Activity

BIAcore Analysis scFv(225.28S) binding to its anti-idiotypic antibody MF-11 was assayed by surface plasmon resonance techniques using a BIAcore machine (Pharmacia) (Jönsson *et al*, 1991). Seven thousand resonance units of purified MF-11 (Kusama *et al*, 1989) were immobilized on a BIAcore microsensor chip at pH 4.8 using standard NHS-EDC chemistry. Binding was clearly observed with scFv(225.28S)-tag (tag = Cys-myc, P-FLAG, or CAL) supernatants and purified preparations, but not with supernatants containing scFv(D1.3) and scFv(HyHEL-10) antilysozyme antibodies (Neri *et al*, 1995b) used as negative controls.

Affinity Constant Determination Affinity constants for the 225.28S IgG molecule and for the $F(ab)_2$, Fab, and scFv-Cys-myc fragments toward the high molecular weight melanoma-associated antigen (HMW-MAA) were determined on COLO-38 cells with the Second Antibody Binding (SAB) method (Mariani *et al*, 1987). A microtiter plate was blocked with PBS-gelatin 1% (200 μl per well) for 3 h at 37°C. COLO38 melanoma cells (2.5×10^5) washed in PBS were then added to each well in a total volume of 200 μl. The plate was centrifuged (3 min, 1500 rpm) and the supernatant removed. Antibodies at different concentrations were added to different wells and incubated for 2 h at room temperature. The plate was washed five times with PBS/0.01% Tween-20, after which 100 μl of ^{125}I-labeled protein LG (150,000 cpm per well; specific activity 10–15 μCi per μg) were added to each well and incubated overnight at 4°C. The plate was finally washed five times with PBS/0.01% Tween, and the wells were cut and counted in a gamma counter. The counts obtained were elaborated with the SAB program (Mariani *et al*, 1987). SAB curves were built using the four-parameter logistic function, and the affinity constant was evaluated as the reciprocal of the antibody concentration giving 50% saturation of binding.

Immunohistochemical Analysis Surgical biopsies of normal tissues and melanocytic lesions were obtained from the Surgical Pathology section of the Regina Elena Cancer Institute. Tissue samples were snap-frozen in liquid nitrogen. From each specimen 4-μm cryostat sections were obtained and fixed in absolute acetone for 10 min. Sections were either immediately used in immunohistochemical assays or kept frozen at −20°C (with no loss of serologic activity). Fixed sections stained with 1% toluidine blue were used to evaluate the histologic features of the lesions. The indirect immunoperoxidase stain, aimed at evaluating the ability of scFv antibody fragments to recognize the HMW-MAA expressed *in vivo*, was performed as follows. Sections were incubated sequentially for 1 h with scFv(225.28S)-Cys-myc (10 μg per ml), MAb 9E10 to *c-myc* peptide (10 μg per ml), and biotin-labeled anti-mouse immunoglobulin antiserum. After the last incubation, the immune reaction was detected with a commercially available avidin-biotin system (Vectastain Elite, Burlingame, CA). The enzymatic activity was developed using 3-amino-9-ethylcarbazole as chromogenic substrate for 8 min. Slides were counterstained with Mayer's hematoxylin. Slides on which the incubation with the scFv(225.28S) was omitted were used as negative control. For comparison, the immunohistochemical reactivity of MAb 225.28S was evaluated in parallel on the same surgical biopsies by the same method.

Immunohistochemistry of scFv(225.28S) Binding to COLO38 Cells COLO-38 cells, expressing the HMW-MAA on their membrane, and HT29 (negative control; not shown) were grown on microscope glass cover slips in RPMI medium + 10% fetal bovine serum. When the cells had grown almost to confluence, the cover slips were washed with PBS and incubated with (i)

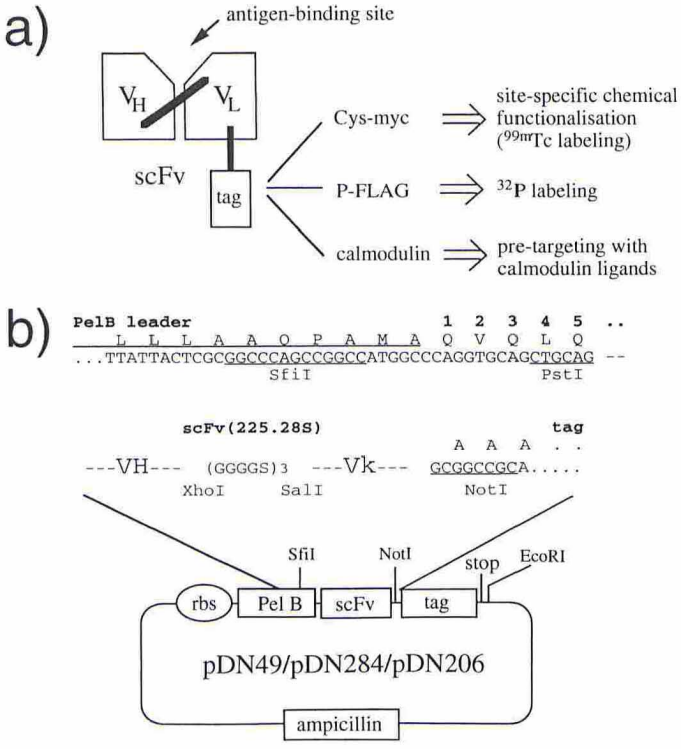

Figure 1. The schematic structure of scFv(225.28S) with convenient sites for antibody modification. *a)* Schematic representation of the VH and VL domains of the 225.28S antibody assembled in scFv configuration. The sequences of the three tags are in *Materials and Methods*. *b)* Scheme of the expression vectors for scFv(225.28S); rbs, ribosome binding site; PelB, leader sequence; VH and Vk, immunoglobulin variable domains of scFv(225.28S); $(GGGGS)_3$, hydrophilic 15-mer linker; stop, two stop codons. Relevant restrictions sites and amino acid sequences are indicated. *c)* Nucleotide and amino acid sequence of the VH and Vk genes of scFv(225.28S).

scFv-CAL (10 μg per ml; antibody: 225.28S or HyHEL-10 (Neri *et al*, 1995a) as negative control), anti-CAL human Fab DN169/F3 (Neri *et al*, 1995a), and anti-human C_k-FITC (Sigma), or (ii) scFv-P-FLAG (10 μg per ml; antibody: 225.28S or HyHEL-10 (Neri *et al*, 1996a) as negative control), anti-FLAG M2 antibody (Kodak IBI), and FITC-labeled rabbit anti-mouse IgG (Sigma), or (iii) IgG (10 μg per ml; 225.28S or 9E10 as negative control), FITC-labeled rabbit anti-mouse IgG (Sigma), and analyzed with an MRC-600 confocal laser microscope. Images were acquired and printed under identical conditions.

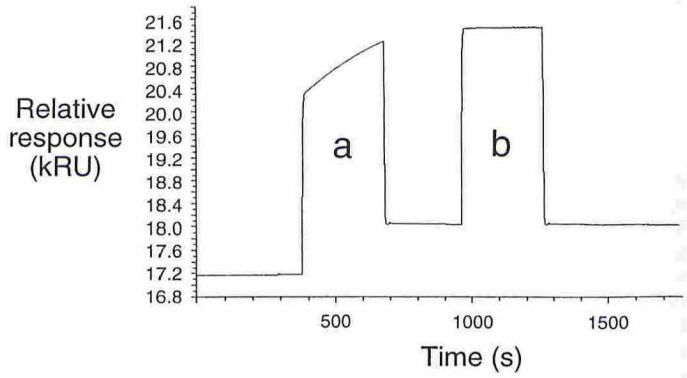

Figure 2. The binding of scFv(225.28S) to its anti-idiotypic antibody MF-11 confirms the successful conversion of IgG(225.28S) in scFv format. BIAcore analysis of scFv(225.28S) binding to a microsensor chip coated with 7000 resonance units of the anti-idiotypic antibody MF-11 (Kusama *et al*, 1989). *a*, The sensogram shows the binding of the recombinant antibody supernatant to MF-11, followed by a flat (i.e., very slow) dissociation profile with signal clearly above the baseline. The vertical increase and decrease in signal at the beginning and at the end of antibody injection are due to refractive index changes. The shape of the association curve indicates that the surface is not saturated. *b*, A noncorrelated anti-lysozyme antibody supernatant does not bind; the baseline remains at the same level before and after injection.

RESULTS

ScFv(225.28S) Can Be Expressed, Purified, and Modified with Different Tags Starting from the 225.28S hybridoma, we cloned the scFv(225.28S) with the conventional $(Gly\text{-}Gly\text{-}Gly\text{-}Gly\text{-}Ser)_3$ 15-mer linker as described (Clackson *et al*, 1991). The VH and VL gene sequences, reported in **Fig 1*c***, coincide with those obtained from the 225.28S hybridoma. The colonies obtained were grown for soluble antibody expression, and positive clones were identified by surface plasmon resonance analysis with a BIAcore machine (Pharmacia), using the anti-225.S28 anti-idiotypic antibody MF-11 (Kusama *et al*, 1989) as probe **(Fig 2)**. Specific binding

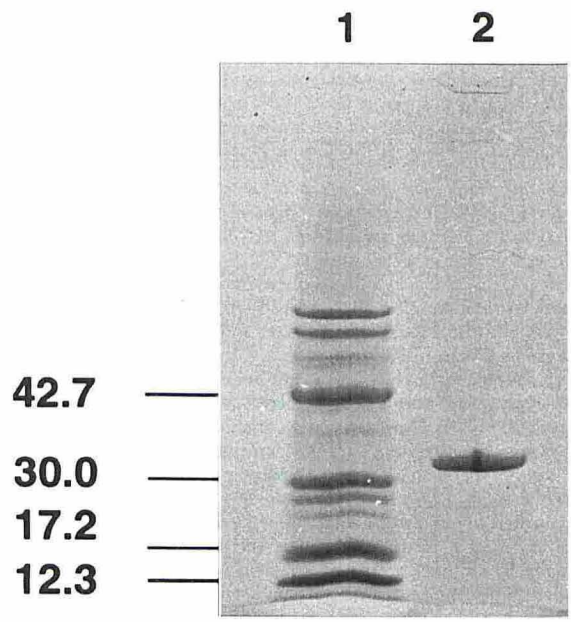

Figure 3. ScFv(225.28S) can be affinity-purified to homogeneity. Polyacrylamide gel electrophoresis analysis of 9E10-purified scFv(225.28S)-Cys-myc. Molecular weight markers are indicated.

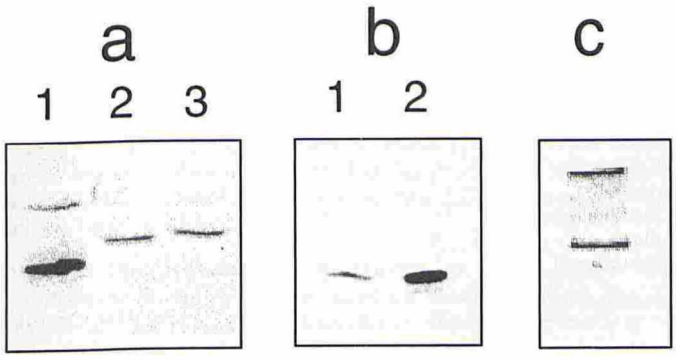

Figure 4. Different formats of scFv(225.28S) can be modified covalently or noncovalently. *a*, 20% SDS-Phast Gel (Pharmacia) of scFv(225.28S)-Cys(fluorescein)-myc, imaged with the cooled-CCD camera-based fluorescent gel imager LUANA (Kinetic Imaging, Liverpool, UK,, and Digital Pixel, Brighton, UK). *Lane 1*, BSA-FITC (60 kDa) + Soybean Trypsin Inhibitor-FITC (20 kDa); *lane 2*, scFv(225.28S)-Cys(fluorescein)-myc; *lane 3*, scFv(D1.3)-Cys(Fluorescein)-myc. *b*, Molecular Dynamics (Sunnyvale, CA) PhosphorImager autoradiogram of a 20% SDS-Phast Gel (Pharmacia). *Lane 1*, ^{32}P-labeled scFv(HyHEL-10)-P-FLAG; *lane 2*, ^{32}P-labeled scFv(225.28S). *c*, 20% Native Phast Gel (Pharmacia) of scFv(225.28S)-CAL in the complex with 1 μM calmodulin-binding TAG3-fluorescein, imaged with LUANA. Experimental conditions as described (Neri *et al*, 1995a). The *black band* at the top of the gel corresponds to the interface between stacking and running gel in Pharmacia Phast gels.

Table I. Bivalent 225.28S Antibody Formats Exhibit High Apparent Affinity Toward COLO-38 Cells

Antibody format	K_a^{app} [a] (M^{-1})
IgG	1.0×10^{10}
$F(ab)_2$	1.0×10^{10}
Fab	7.7×10^{8}
scFv-cys	4.8×10^{8}

[a] Apparent affinity constants, displaying avidity effects as a function of antibody valency (Crothers and Metzger, 1972), were measured with a SAB protocol (Mariani *et al*, 1987).

is observed with scFv(225.28S), but not with the anti-lysozyme scFv(HyHEL-10) (Neri *et al*, 1995b).

For facile chemical modification of our anti-melanoma antibodies, we cloned the scFv(225.28S) gene into suitable expression vectors, yielding vectors pDN49, pDN284, and pDN206, which append different tags at the C-terminal extremity (**Fig 1** and *Materials and Methods*). pDN49 appends a cysteine residue followed by a peptide tag (myc-tag; Munro and Pelham, 1986) useful for protein purification and detection. The thiol group of the cysteine residue allows site-specific and stoichiometrically defined functionalization of the recombinant antibody with thiol-specific reagents at a position that does not interfere with antigen binding (Cumber *et al*, 1992; Neri *et al*, 1995b) and does not result in loss of immunoreactivity (for a review, Neri *et al*, 1995c). pDN284 appends to the

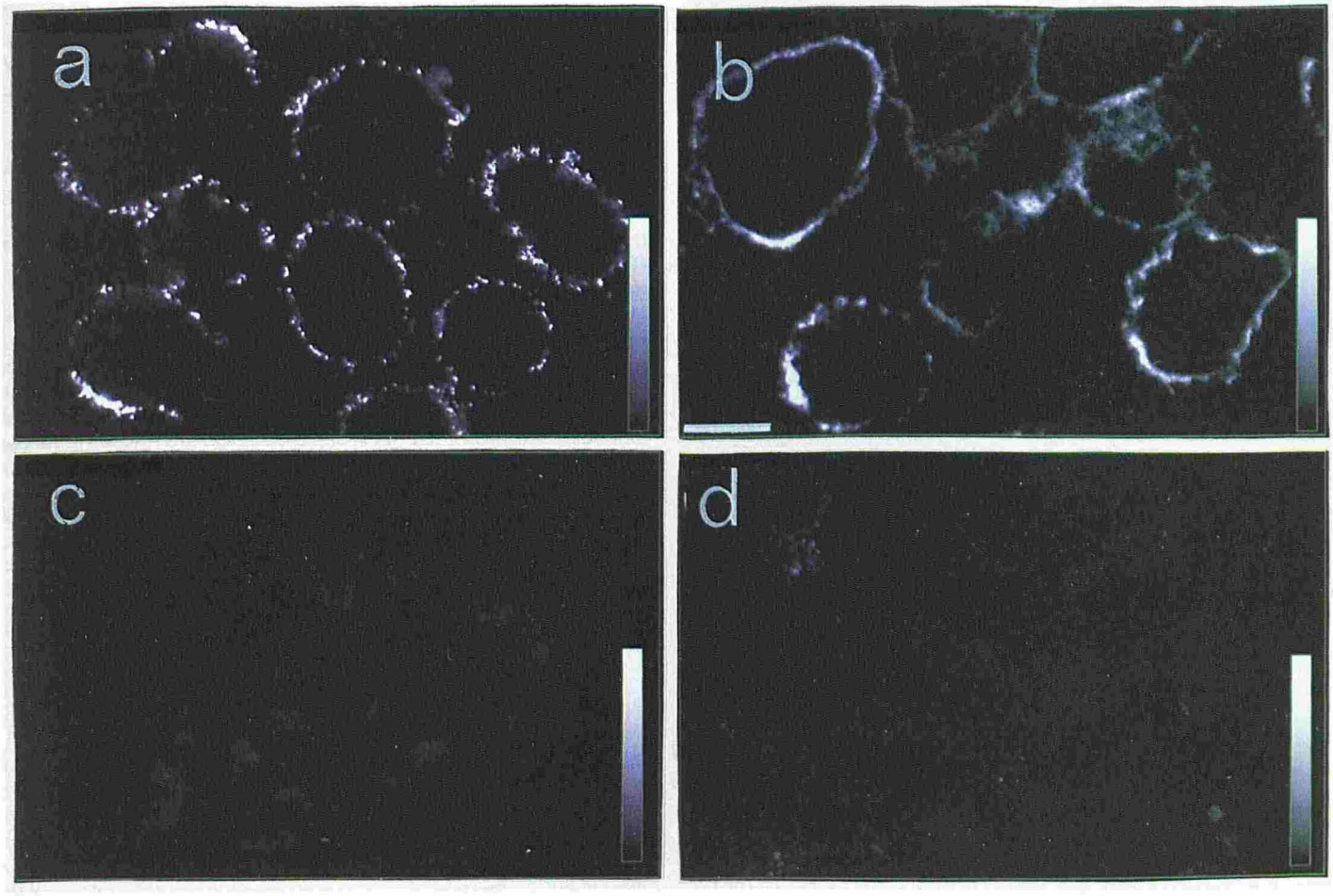

Figure 5. ScFv(225.28S) binds specifically to melanoma cells. Confocal laser microscope analysis of antibody binding to COLO-38 melanoma cells; *a*, scFv(225.28S)-P-FLAG; *b*, IgG(225.28S); *c*, scFv(HyHEL-10)-P-FLAG; *d*, IgG(9E10).

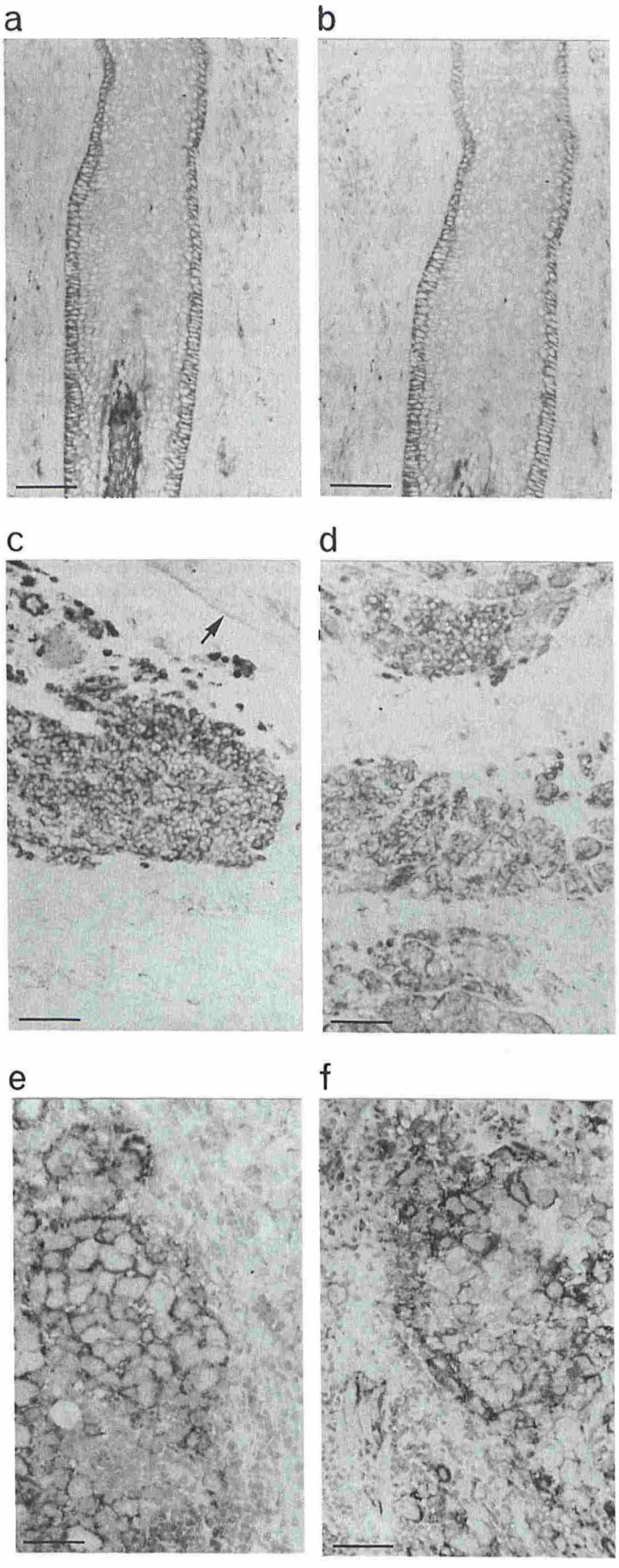

Figure 6. ScFv(225.28S) and MAb(225.28S) stain melanocytic lesions, but not normal tissues. Reactivity was revealed by avidin-biotin indirect immunoperoxidase staining on 4-μm acetone-fixed cryostat sections. Sections *a, c,* and *e* were stained with IgG(225.28S) while *b, d,* and *f* were stained with scFv(225.28S). Both reagents react with the keratinocytes of hair follicle (*a, b*) and with nevus cells (*c, d*). Whereas MAb(225.28S) intensely decorates all melanoma cells of a metastatic lesion (*e*), scFv(225.28S) stains all melanoma cells but with heterogeneous intensity (*f*). *a–d) Scale bar,* 35 μm; *e* and *f), scale bar,* 20 μm. The → in *c* marks the epidermis.

Table II. Melanocytic Lesions Are Stained Specifically by Both scFv(225.28S) and IgG(225.28S)

Normal tissues	scFv	IgG
Cutis	Basal keratinocytes	Basal keratinocytes
Liver	–	–
Thyroid	–	–
Parotid	–	–
Colon-rectum	–	–
Kidney	–	–
Vascular wall	+	+
Melanocytic lesions		
Nevocellular nevi	5/5	5/5
Primary melanomas	4/4	4/4
Metastatic melanomas	6/6	6/6

antibody C terminus a peptidic tag, which can be efficiently phosphorylated by casein kinase II and [γ-^{32}P]ATP to very high specific activity (Neri *et al*, 1996a), and which can be detected with anti-FLAG antibodies (Hopp *et al*, 1988). The ^{32}P-labeled antibody is stable in serum for at least 48 h at 37°C. pDN206 appends CAL at the C-terminal extremity of the recombinant antibody. Several ligands, both peptidic and nonpeptidic in nature, bind to CAL with nanomolar affinity or better, and the CAL moiety of the chimeric antibody can therefore be used for targeting experiments (Neri *et al*, 1995a). On the basis of the structure of the tag used for pDN49, pDN284, and pDN206, we will refer to the corresponding scFv antibody constructs as scFv-Cys-myc, scFv-P-FLAG, and scFv-CAL, respectively.

We purified our scFv-Cys-myc, scFv-P-FLAG, and scFv-CAL as previously described (*Materials and Methods*; Neri *et al*, 1995a, 1995b, 1996a). **Figure 3** shows an sodium dodecyl sulfate-polyacrylamide gel of purified scFv-P-FLAG, illustrating the degree of purity of the antibody preparation after a single purification passage. Typical yields were 1–10 mg per liter of culture.

We tested the functionalization of scFv-Cys-myc with a thiol-specific reagent, iodoacetamidofluorescein. The resulting fluorescently labeled antibody was analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (Laemmli, 1970) using the fluorescence gel-imager LUANA (Neri *et al*, 1996b) for detection **(Fig 4*a*)**. Similarly, scFv(225.28S) could be site-specifically biotinylated with iodoacetamido-LC-biotin. ScFv-P-FLAG phosphorylation was performed as described (Neri *et al*, 1996a), using casein kinase 2 and [γ-^{32}P]ATP. **Figure 4*b*** shows the autoradiogram of a sodium dodecyl sulfate-polyacrylamide gel electrophoresis with the labeled product and, for control, a ^{32}P-labeled anti-lysozyme antibody. Targeting of scFv-CAL by CAL-binding peptides is demonstrated in **Fig 4*c***. ScFv-CAL was incubated with a fluorescein-labeled CAL-binding peptide, TAG3, as described (Neri *et al*, 1995a), and the resulting mixture run on a Pharmacia Homogeneous 20 Phast gel with native buffer strips. A strong fluorescent band associated with the complex was detected using the luminescence analyser LUANA (Neri *et al*, 1996b).

Bivalent Anti-Melanoma Antibodies Have Higher Functional Affinity than Monovalent Fragments Apparent affinity constants (K_a^{app}) for COLO-38 cells for the 225.28S antibody in different formats were measured using the SAB method (Mariani *et al*, 1987; *Materials and Methods*). SAB curves were built using the four-parameter logistic function; the affinity constant was evaluated

as the reciprocal of the antibody concentration giving 50% saturation of binding. K_a^{app} for 225.28S in IgG, $F(ab)_2$, Fab, and scFv-Cys-myc format are listed in **Table I**. The increase (from 0.5 nM to 100 pM levels) of apparent affinity for bifunctional antibodies [IgG, $F(ab)_2$] is attributable to increased avidity (Crothers and Metzger, 1972). An implication of these results is that bi- or multivalent recombinant antibody formats (Pack *et al*, 1993) should be preferable for therapeutic use of the 225.28S antibody and optimal binding to melanomas.

ScFv(225.28S) Retains the Specificity of the Parental IgG

In addition to binding to the anti-idiotypic antibody MF-11 **(Fig 2)**, scFv(225.28S)-Cys-myc was assayed for HMW-MAA binding on the melanoma-derived COLO-38 cell line. A radioimmunoassay using iodinated protein LG as detecting reagent (Kihlberg *et al*, 1992; Vola *et al*, 1994) showed that the total radioactivity delivered by scFv-Cys-myc and $F(ab)_2$ to COLO-38 cells is comparable (data not shown). The immunoreactivity of scFv-Cys-myc is not lost upon biotinylation, as is expected because of the mild and site-specific functionalization reaction (**Fig 1;** *Materials and Methods*).

The binding of scFv(225.28S)-CAL, scFv(225.28S)-P-FLAG, or IgG(225.28S) to COLO-38 melanoma cells and to HT29 cells (negative control; not shown) was analyzed by confocal laser microscopy using a human anti-CAL Fab fragment (Griffiths *et al*, 1994; Neri *et al*, 1995a). The results, shown in **Fig 5** for COLO-38 cells targeted with scFv(225.28S)-P-FLAG and IgG(225.28S), confirm that the specificity of the parental antibody is conserved in the recombinant antibody constructs used.

The immunohistochemical reactivity of scFv reagent on normal and transformed tissues is summarized in **Table II**. The immunohistochemical analysis revealed that both scFv and IgG stained all melanoma cells. The staining by IgG was intense and homogeneous; the scFv yielded weaker and more variable staining. Examples of these immunohistochemical findings are shown in **Fig 6**.

DISCUSSION

The development of murine MAbs to tumor-associated antigens has resulted in an increasing effort to employ these reagents and their proteolytic fragments for diagnosis and therapy. At present, no general criteria for the selection of antibody-based pharmaceuticals have been defined, even though it is generally agreed that parameters such as antibody affinity, valency, specificity, clearance, and penetration are of great importance.

With the advent of recombinant DNA technology and of the polymerase chain reaction, it has become possible to rescue antibody genes by polymerase chain reaction from hybridomas and to express antibody fragments in bacteria (Skerra and Plückthun, 1988; McCafferty *et al*, 1990; Winter and Milstein, 1991). Recombinant antibodies are typically expressed either as Fab fragments or as scFv fragments. Antibodies in scFv configuration penetrate tissues more effectively than conventional antibodies and have a more rapid clearance (Yokota *et al*, 1993). Their small size makes them less likely to be immunogenic.

ScFv production in bacteria is economical and lends itself well to industrial production. Even more important, the requirements for the use of recombinant antibodies in pilot clinical trials are easily met by academic laboratories (Begent *et al*, 1993). This should contribute to overcoming one of the major obstacles for the development of novel immunopharmaceuticals using hybridoma technology: the high cost of production of a cell bank, which often prevents interesting molecules from being investigated in the clinic.

Recombinant antibody modification by protein engineering techniques is particularly easy at the gene level. This can provide recombinant antibodies with "designer" effector functions. For example, several types of chimeras between recombinant antibodies and other proteins have been obtained by sequentially fusing the corresponding genes (for a review, Neri *et al*, 1995c).

It has recently become possible to obtain high-affinity recombinant antibodies from phage display libraries (Griffiths *et al*, 1994; Nissim *et al*, 1994) without immunization. Hybridomas producing well-established antibodies, however, still represent invaluable starting material for obtaining recombinant antibodies.

We have reported here the cloning and characterization of the anti-melanoma 225.28S antibody in constructs suitable for bacterial expression with different effector functions. We have cloned a recombinant scFv fragment antibody with specificity identical to that of the parental IgG(225.28S) molecule. The recombinant antibody can be produced in good yield by bacterial fermentation and is easy to purify.

Three expression vectors, appending three different tags at the C terminus of the scFv(225.28S), have been developed, which allow the covalent and noncovalent modification of the recombinant antibody. By site-specific chemical modification of scFv(225.28S)-Cys-myc, the antibody can be armed with biologically useful effector functions. A mild and stoichiometrically defined recombinant antibody conjugation to toxins, pro-drug converting enzymes, radionuclide chelators, biotin, and other molecules (for a review, see Neri *et al*, 1995c) is possible. Direct antibody labeling with ^{99m}Tc at the free thiol group of the cysteine residue (Dean *et al*, 1990; Liberatore *et al*, 1995) may be relevant for immunoscintigraphic applications. Furthermore, the same preparation of scFv(225.28S) can be functionalized with different reagents and tested for therapeutic performance.

The facile, stable, and relatively inexpensive ^{32}P labeling of scFv-P-FLAG with casein kinase II and [γ-^{32}P]ATP opens the way to possible radiotherapeutic applications of scFv(225.28S). ^{32}P is considered an excellent nuclide because of its physical properties and because its half-life (14.3 d) matches the half-life of antibodies on tumors (Britton *et al*, 1991; Howell *et al*, 1994). Toward this goal, however, a detailed immunoscintigraphic investigation of scFv(225.28S) is still required.

CAL-tagged scFv(225.28S) can be produced and purified efficiently. Targeting with high-affinity functionalized peptidic or nonpeptidic CAL binders (Neri *et al*, 1995a; Montigiani *et al*, 1996) should allow multistep immunoscintigraphic protocols, similar to what has already been described for the biotin-avidin system (Paganelli *et al*, 1991), but with the possible advantage of the low immunogeneicity (VanEldik and Lukas, 1987), and small size of CAL. CAL ligands with kinetic association constants k_{on} greater than 10^8 1/sM and kinetic dissociation constant k_{off} lower than 10^{-5} 1/s are available (Török and Trentham, 1994; Neri *et al*, 1995a; Montigiani *et al*, 1996). Pre-targeting strategies offer in principle the possibility of experimentally varying several parameters such as the amount of antibody to be injected, type of targeting molecule, affinity of pre-targeting binding reaction, and time of administration.

Avidity effects (Crothers and Metzger, 1972) are likely to play an important role in the performance of several constructs of binding 225.28S antibody to cells. Functional affinity measurements for different antibody constructs for 225.28S have shown that IgG and $F(ab)_2$ perform better than Fab and scFv. For *in vivo* applications, it may be important to append dimerization or multimerization domains to scFv(225.28S) (Pack *et al*, 1993; King *et al*, 1994) in order to increase antibody residence time on tumors without affecting its residence time in the blood. This would be particularly important for therapy with long-lived isotopes such as ^{32}P.

In conclusion, we have developed different formats for the bacterial expression of recombinant anti-melanoma 225.28S. These functional recombinant proteins should prove valuable as "building-blocks" for melanoma targeting.

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