Identification of Human scFvs Targeting Atherosclerotic Lesions

SELECTION BY SINGLE ROUND IN VIVO PHAGE DISPLAY

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Our aim was to investigate by in vivo biopanning the lesions developed early in atherosclerosis and identify human antibodies that home to diseased regions. We have designed a two-step approach for a rapid isolation of human Monoclonal phage-display single-chain antibodies (MoPhabs) reactive with proteins found in lesions developed in an animal model of atherosclerosis. After a single round of in vivo biopanning, the MoPhabs were eluted from diseased sections of rabbit aorta identified by histology and NMR microscopy. MoPhabs expressed in situ were selected by subtractive colony filter screening for their capacity to recognize atherosclerotic but not normal aorta. MoPhabs selected by our method predominantly bind atherosclerotic lesions. Two of them, B3.3G and B3.GER, produced as scFv fragments, recognized an epitope present on the surface in early atherosclerotic lesions and within the intimal thickness in more complex plaques. These human MoPhabs homed to atherosclerotic lesions in ApoE −/− mice after in vivo injection. A protein of ~56 kDa recognized by B3.3G was affinity-purified and identified by mass spectrometry analysis as vitronectin. This is the first time that single round in vivo biopanning has been used to select human antibodies as candidates for diagnostic imaging and for obtaining insight into targets displayed in atherosclerotic plaques.

Atherosclerosis is an inflammatory disease in which the immune system interacts with risk factors to initiate, propagate, and activate lesions in the vasculature that are referred to as atherosclerotic plaques (1, 2). The rupture of such plaques and subsequent thromboembolism lead to cardiovascular disease, the major cause of death in Western countries. The early detection of atherosclerosis and the development of “smart” contrast agents to be used in a non-invasive procedure such as magnetic resonance imaging are critical for the diagnosis and management of vulnerable patients. An early event in plaque formation is the interaction of circulating platelets with activated endothelial cells (3) with ensuing leukocyte recruitment via soluble or cell surface signaling molecules such as proinflammatory cytokines and selectins. This cascade of events including the tight adhesion of platelets leads to local release of potent inflammatory mediators (IL1B and CD40L) at the injured site. The endothelial cells then show an enhanced expression of adhesion molecules including VCAM-1, I-CAM, and P-selectin as well as the release of MCP-1 and other chemokines. Their altered chemotactic and adhesive properties facilitate lymphocyte and monocyte transmigration into the intima of blood vessels. The “signature” provided by an atherosclerotic lesion surface is difficult to study in vitro (4), even if progress in cell co-culture procedures allow mimicking of the disease (5). An alternative for overcoming this limitation is to probe the lesions in a high cholesterol fed rabbit model using a human phage-displayed single chain antibody library. Peptide phage display libraries have been particularly useful for the analysis and the isolation of amino acid sequences that target tissue or disease-affected endothelial cell (6–8). However, to our knowledge, selection by in vivo phage display of human scFvs against atherosclerotic plaques has never been reported in the literature, despite the fact that human antibodies are the “gold standard” as clinical reagents both for diagnosis and for therapy (9).

In our study, we have combined in vivo biopanning of a monoclonal phage-display single-chain antibody (MoPhab) library and a high throughput screening procedure. After only one round of selection to avoid the loss of scFvs directed against rare epitopes, the eluted recombinant phages expressing mAbs have been secondarily screened by an original subtractive colony filter procedure. Described for the first time by Dreher et al. (10), this method was recently used in combination with a robot picker for screening thousands of clones simultaneously (11). Filter screening methods offer many advantages compared with classical random picking (12, 13). Following these guiding principles (11), we have selected two scFvs, B3.3G and B3.GER, that

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‡ The abbreviations used are: MoPhab, monoclonal phage-displayed single-chain antibody; mAb, monoclonal antibody; ELISA, enzyme-linked immunosorbent assay; Amp, ampicillin; Kan, kanamycin; PBS, phosphate-buffered saline; MPBS, milk-phosphate-buffered saline; pfu, plaque-forming unit; MS, mass spectrometry; MS/MS, tandem MS.
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specifically recognize epitopes expressed by atherosclerotic lesions. A protein of ~56 kDa recognized by B3.3G was affinity-purified and identified by mass spectrometry analysis as vitronectin. ScFv human antibody fragments are potential vectors for directing therapeutic agents to injured sites or for non-invasive imaging after coupling with appropriate contrast agents (14, 15).

**EXPERIMENTAL PROCEDURES**

**Animals**—The investigation conformed to the Guide for the Care and Use of Laboratory Animals published by the United States National Institutes of Health (NIH Publication No. 85-23, revised 1996), and the local ethics committee for animal experiments approved our procedures. Adult male Bourgogne brown rabbits were submitted for 8 months to an atherogenic diet including 0.3% cholesterol. Some of them were subjected to an angioplasty of the aorta (with a 3F balloon) under anesthesia by intramuscular administration of 20 mg/kg ketamine and 2 mg/kg xylazine. The denudation was performed 5 weeks and 1 week before sacrifice to allow the formation of complex plaques with intramural thrombi.

Homozygous ApoE gene-inactivated (knock-out) mice were from a backcross onto a C57BL/6J background and were obtained from Charles River Laboratories (St Germain sur l’Arbresle, France). They were given a 0.25% cholesterol diet for 16 weeks.

**Tomlinson I + J Phage Libraries**—The recombinant scFvs were issued from two different human antibody libraries (Library I and Library J provided by MRC Geneservice). Both libraries are based on a single human framework for VH (V3–23/DP-47 and J14/Ab) and Vx (O12/O3/DPK9 and Je1) with side chain diversity (either DVT or NNK encoded) incorporated in complementary determining region 2 and complementary determining region 3 at positions in the antigen binding site that make contacts to antigen in known structures and are highly diverse in the mature repertoire. The libraries I and J are constructed in the pIT2 vector (derived from pHEN1), and the library size is about $1.4 \times 10^8$ different scFv fragments.

**Phage Library Preparation**—Phage libraries were inoculated in 50 ml of 2TY medium (16 g/liter tryptone, 10 g/liter Bactoyeast extract, 5 g/liter NaCl, pH 7) + 100 μg/ml ampicillin (Amp) + 100 mM glucose to $A_{600} = 0.03$ at 37 °C and shaken at 250 rpm. When an $A_{600} = 0.4$ was reached, the culture was infected with $2 \times 10^{11}$ M13KO7 helper phage (Invitrogen, Cergy-Pontoise, France) and incubated at 37 °C for 30 min without shaking, followed by 30 min at 250 rpm. The cells were next harvested by centrifugation for 10 min at 3000 x g and the pellet was re-suspended in 50 ml of 2TY + 100 μg/ml Amp + 50 μg/ml kanamycin (Kan) + 100 μg/ml isopropyl-1-thio-β-D-galactopyranoside. The MoPhabs were produced at 30 °C with agitation at 250 rpm overnight. Bacteria were pelleted by centrifugation for 10 min at 3000 x g. MoPhabs in the supernatant were precipitated with 0.20 volume of 20% PEG/2.5 M NaCl solution for 6 h on ice with gentle shaking and pelleted for 45 min at 15,000 x g at 4 °C. The MoPhabs were re-suspended in 1 ml of sterile cold PBS and cleared of residual cell debris by an additional centrifugation at 10,000 x g for 10 min.

**SDS-PAGE and Western Blot**—MoPhabs were subjected to 10% SDS-PAGE and transferred onto polyvinylidene difluoride membranes. The membrane was blocked with 5% milk-phosphate-buffered saline (MPBS) for 2 h at room temperature. The g5p minor coat protein was detected with a mouse anti-g5p mAb (1:1000) (Mobitec, Gottingen, Germany) for 2 h at room temperature. After three washings with PBS, 0.1% Tween 20 buffer (PBST), horseradish peroxidase-conjugated goat anti-mouse IgG (Jackson ImmunoResearch, West Grove, PA) (1:5000) was used for detection and visualized by ECL (Pierce, Brebières, France).

**In Vivo Biopanning**—The phage libraries (I+J) were mixed, and $10^{13}$ pfu were injected intravenously under anesthesia in the rabbit marginal ear vein in a total volume of 2 ml of PBS. After 2 h, the animal was sacrificed by an overdose of anesthetic and perfused via the heart with 200 ml of PBS to wash out the bound phages. The aorta was then removed from the aortic arch to the iliac bifurcation, washed, and divided into several sections corresponding to: the aortic arch, the superior thoracic aorta, the inferior thoracic aorta, the superior abdominal aorta, and the inferior abdominal aorta.

The bound phages were eluted using a two-step method. After extensive washing in PBS, the aortic segments were weighed, snap-frozen in liquid nitrogen, and hand-homogenized in a mortar under liquid nitrogen. All the following procedures were performed on ice. MoPhabs associated with surface lesions were first eluted using 500 μl of 0.1 M glycine, pH 2.2, for 15 min and neutralized with 50 μl of 2 M Tris-HCl, pH 8. A mixture of protease inhibitors was added at a dilution recommended by the manufacturer (Sigma). Phages were recovered after a centrifugation step ($5000 \times g$, 10 min at 4 °C). RPMI medium supplemented with 20% radioimmunoprecipitation assay buffer (10 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.1 mM EDTA, 1% Nonidet P-40), and the same mixture of protease inhibitors was used to re-suspend the pellet that was homogenized 30 s (four pulses) with a Polytron TP-20 homogenizer (Kinematica, Luterne, Switzerland). Twenty ml of XXI-Blue bacteria (Stratagene, La Jolla, CA) in log phase were added (i) to the 0.1 M glycine isolated phages and (ii) to the tissue homogenate, for 15 min without shaking to allow infection with eluted phages and 45 min with shaking. Phages were then titered by serially diluting the infected bacteria in 2TY medium and plating them out in parallel on Amp and Kan agar plates for incubation overnight at 37 °C.

**Histology and NMR Microscopy Imaging of Aortic Sections**—Segments near the sections to be eluted were excised and immersed in buffered paraformaldehyde for immediate NMR microscopy imaging at 9.4 tesla. FLASH sequence parameters were: echo time/repetition time, 2.6 ms/12 ms; angle, 15°; acquisitions, 12; field of view, $10 \times 9.9 \times 9.9$ mm; matrix, $128 \times 110 \times 110$; resolution, $78 \times 90 \times 90$ μm; total scan time, 30 min. The embedded segments of the aorta were further sectioned (8 μm) and, after deparaffinization, processed for staining with Masson’s trichrome for histological examination of neutral lipid, thrombi, fibrous, and cellular components. Sections from each segment were examined for lesion formation using a light microscope (Nikon Microphot Fx).

**Protein Extraction and Coating onto Nitrocellulose Filters**—Aortas from rabbits fed a normal or an atherogenic diet but which did not receive recombinant phages were removed and snap-
frozen in liquid nitrogen. The aortas were weighed and homogenized in a mortar in liquid nitrogen. The powder was incubated at 4 °C for 1 h in 1 ml of radioimmunoprecipitation assay buffer in the presence of protease inhibitors. Further disruption was performed using a Polytron homogenizer. Homogenates were then centrifuged at 20,000 × g at 4 °C for 30 min to collect the supernatant containing proteins whose concentration was measured using the Bradford assay (Pierce, Perbio Science). SDS-PAGE followed by Coomassie Blue staining was used to assess the extraction of proteins from atherosclerotic (Fig. 1a) and normal aorta (Fig. 1b). The two antigen-coated filters (atherosclerotic protein-coated filter and normal aorta protein-coated filter) were prepared as follows. Briefly, two 80-mm nitrocellulose membranes (Protran BA85, Schleicher and Schuell, Dassel, Germany) were coated overnight at 4 °C in 15 ml of PBS containing 50 μg/ml protein extract of each. Coating efficiency was evaluated histochemically using a mouse mAb (RAM-11) directed against rabbit macrophase IgG receptor (16) on filters containing proteins from atherosclerotic plaques (Fig. 1c) or from normal aorta (Fig. 1d). RAM-11 was detected by an enzymatic diaminobenzidine colorimetric reaction using an anti-mouse IgG-biotinylated antibody followed by peroxidase-labeled streptavidin. The helper phage was used as a negative control (Fig. 1, black arrows). The two filters were then incubated with an anti-g8p (anti-M13) mAb for 3 h at 37 °C. After three washes in PBST (0.1%), a third incubation was carried out with a biotinylated antibody to mouse IgG diluted 1/1000 (Jackson ImmunoResearch) followed by peroxidase-labeled streptavidin diluted 1/5000. Bound MoPhabs were detected by chemiluminescence using ECL Western blotting detection reagents (Pierce). The spots giving a signal on the atherosclerotic protein filter (see Fig. 1g, black arrows) with no labeling of the normal aorta protein filter (Fig. 1h) were used to identify the corresponding colonies on the master plate. The selected clones responsible for the production of specific MoPhabs were removed with a toothpick, inoculated in 5 ml of 2TY medium + 100 μg/ml Amp + 100 mM glucose, and grown overnight at 37 °C for further plasmid preparations.

**PCR and Restriction Analysis of Selected Clones**—The scFv DNA inserts of selected clones were amplified by a PCR performed directly on colonies using pIT2 primers: LMB3 (5’-CAGGAAAACAGTATGAC-3’) and pHENseq (5’-CTAT-GCGGCCCCATCCA-3’). One μg of the plasmid preparations was digested with restriction enzymes NcoI/NotI (Promega) for 1 h at 37 °C. PCR and digestion products were analyzed on 1% agarose gels.

**Phage-scFv Reactivity Determined by ELISA**—Ninety-six-well plates were coated overnight, at 4 °C, with 50 μg/ml protein extracts from atherosclerotic aorta, normal aorta, bovine serum albumin, or 5 μg/ml vitronectin in NaHCO3 buffer, pH 9.6. Bound MoPhabs were labeled with an anti-M13 mAb diluted 1/500 in 0.5% MPBS. After a 2-h incubation, a 1/1000 dilution of horseradish peroxidase-conjugated anti-mouse IgG was added and incubated for 90 min. Color was developed with 2,2’-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) and the absorbance was read at 405 nm with an Emax precision microplate reader (Molecular Devices, Sunnyvale, CA).

**Expression and Purification of Soluble scFv Antibodies**—The scFv genes were cloned into the vector pHOG21 for soluble expression. The scFv genes of selected MoPhabs were digested into pIT2 with NcoI and NotI, separated on agarose gel, and purified. Fractions were next ligated to NcoI/NotI-digested pHOG21. Transformed XL1-Blue E. coli bacteria were grown overnight in 2TY medium supplemented with 50 μg/ml ampicillin and 100 mM glucose at 37 °C. The soluble scFvs were identified in the supernatant with an anti-M13 antibody followed by a biotinylated anti-mouse IgG and peroxidase-labeled streptavidin, followed by a DAB colorimetric reaction. In the subtractive colony filter screening, clones secreting MoPhabs were probed with an anti-M13 antibody followed by a biotinylated anti-mouse IgG peroxidase-labeled streptavidin and chemiluminescence detection. MoPhabs that gave a positive reaction on filters containing proteins from plaques (g) and a negative reaction on filters coated with proteins from control aorta (h) are highlighted with black arrows.

After the soft agarose had solidified, the 2TYAG plates were left for 1 h at 37 °C to allow the helper phage infection. In the meantime, the antigen-coated filters were blocked in 5% MPBS for 1 h at room temperature, washed three times in PBS soaked in 2TY medium, and transferred onto a 2TY + 100 μg/ml isopropyl-1-thio-β-D-galactopyranoside + 100 μg/ml Amp + 50 μg/ml Kana solid medium (2TY AKI). The two bacteria filters covered by the M13KO7-containing agar were then carefully removed with tweezers and replaced on their respective antigen-coated filters on 2TY AKI for overnight MoPhab induction at 30 °C. This medium allowed the selection and the induction of bacteria containing a recombinant pIT2 vector (Amp resistance) and infected by the helper phage (Kan resistance). The produced MoPhabs diffuse from the bacteria filter to the antigen filter placed on top of the solid medium and bind to their respective targets. The next day, the antigen-coated filters were removed and washed three times in PBST (0.1%). The two filters were then incubated with an anti-g8p (anti-M13) mAb for 3 h at 37 °C. After three washes in PBST (0.1%), a third incubation was carried out with a biotinylated antibody to mouse IgG diluted 1/1000 (Jackson ImmunoResearch) followed by peroxidase-labeled streptavidin diluted 1/5000. Bound MoPhabs were detected by chemiluminescence using ECL Western blotting detection reagents (Pierce). The spots giving a signal on the atherosclerotic protein filter (see Fig. 1g, black arrows) with no labeling of the normal aorta protein filter (Fig. 1h) were used to identify the corresponding colonies on the master plate. The selected clones responsible for the production of specific MoPhabs were removed with a toothpick, inoculated in 5 ml of 2TY medium + 100 μg/ml Amp + 100 mM glucose, and grown overnight at 37 °C for further plasmid preparations.

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expressed as described previously (17) by preparing periplasmic extracts on a small scale and purified by immobilized metal affinity chromatography on nickel-nitrilotriacetic acid spin columns (Qiagen, Courtaboeuf, France) according to the manufacturer’s protocol.

Immunohistochemical Analysis on Rabbit and Human Atherosclerotic Sections—Paraffin-embedded sections of aortic tissue were deparaffinized, rehydrated, and endogenous peroxidase blocked with 0.3% H2O2 in methanol. Nonspecific binding of MoPhabs (1011 pfu per section) or diluted scFv preparations were applied for 2 h at room temperature. Following three washes with PBS, a diluted mouse anti-g6p antibody (Amersham Biosciences) (1/500) or anti-c-Myc antibody (1/200) was applied to the sections for 1 h at room temperature. After a further three washes with PBS, staining was performed using a peroxidase-labeled biotin-streptavidin complex (Amersham Biosciences). The substrate for the peroxidase was diaminobenzidine (Vector) with H2O2.

In Vivo Homing of Individual MoPhabs in ApoE Mice—Binding of MoPhab B3.3G was determined in vivo to atherosclerotic lesions developed in ApoE mice. Injections were performed in the tail vein of animals anesthetized via inhaled isofluorane. After 30 min, the mice were perfused with sterile PBS and 4% paraformaldehyde for fixation. The aortas were removed by dissection and cleaned, opened longitudinally, and immobilized in a Petri dish with needles. The endogenous peroxidase activity was inhibited with 3% H2O2 before application of the anti-M13 peroxidase-conjugated antibody (Amersham Biosciences) at 1/1000 dilution. After three washes with PBS, the reaction was developed with diaminobenzidine substrate.

Immunoblot Analysis—Protein extracts from atherosclerotic or from normal aortas were separated by SDS-PAGE and transferred onto nitrocellulose membranes. The membranes were blocked overnight at 4°C with 5% MPBS and incubated with B3.3G-soluble scFv for 2 h at room temperature. After extensive washing, the membranes were successively incubated with mouse monoclonal anti-His tag antibody (Qiagen) and horseradish peroxidase-conjugated goat anti-mouse IgG (Jackson Immunoresearch). Bound scFv were detected by chemiluminescence using ECL Western blotting detection reagents (Pierce).

Immunoprecipitation Experiments—Proteins (1 mg) extracted either from atherosclerotic aorta or from normal aorta were first subjected to a control incubation for 2 h with anti-c-Myc monoclonal antibody coupled to superparamagnetic microbeads (Miltenyi Biotech, Bergisch Gladbach, Germany). After being applied to the magnetic column (Miltenyi Biotech), the flow-through containing the proteins was next subjected to immunoprecipitation. Soluble scFvs with c-Myc inserts were incubated overnight at 4°C under rotation. The next day, scFvs bound to their targets were incubated for 2 h with anti-c-Myc monoclonal antibody coupled to superparamagnetic microbeads. The antigens were then eluted from the magnetic column and separated by SDS-PAGE using 10% gels. Bands that appeared using the atherosclerotic aorta protein extract but not for that of controls (normal aorta protein extract with or without scFv addition) were cut out and subjected to mass spectrometry analysis.

Mass Spectrometry Analysis—Mass spectrometry analysis was performed on the Functional Genomics Plateform of Bordeaux University. The protein band at ~56 kDa was excised after SDS-PAGE, digested in trypsin, and submitted to on-line capillary chromatography (LC Packings, Amsterdam, The Netherlands) coupled to a nanospray LCQ ion trap mass spectrometer (ThermoFinnigan, San Jose, CA). The mass spectrometer was operated in positive ion mode. Data acquisition was performed in a data-dependant mode consisting of, alternatively in a single run, a full scan MS over the range m/z 300–2000 and three full scan MS/MS of the most intense ions in the preceding MS spectra. Proteins were identified by peptide mass mapping using SEQUEST (ThermoFinnigan, Torrence, CA) against the SwissProt data base. A more detailed description of the procedure is described elsewhere (18).

RESULTS

In Vivo Biopanning and Recovery of Bound MoPhabs—We have used a human scFv library in the pIT2 phagemid vector. Before proceeding to the in vivo selection, we confirmed by Western blotting the presence of scFvs fused to the g6p proteins of the phage. We then injected 1013 MoPhabs into the marginal ear vein of a rabbit fed with a high cholesterol diet and with advanced atherosclerotic plaques. The MoPhabs were allowed to circulate for 2 h. To minimize the selection against immunodominant epitopes expressed in atherosclerotic lesions and to retain the diversity of binders, only a single round of selection was performed. A 2-h circulation also permitted entry of MoPhabs within the plaque. After animal sacrifice and abundant washes, the aorta was harvested and ring sections surrounding the MoPhab elution area were analyzed by histology and ex vivo NMR microscopy imaging to check for the presence of atherosclerotic plaques. A two-step elution protocol was used. After mechanical homogenization of the aorta, bound phages were first eluted at acidic pH and then neutralized (supernatant fraction). Aorta homogenate were next disrupted mechanically several times in a soft lysis buffer for recovery of internalized phages (pellet fraction). We have recovered 1.6 × 107 phages/g of tissue in the supernatant fraction, while 3 × 106 phages/g were recovered in the pellet (internalized phases).

Histological Analysis and NMR Microscopy Imaging—To confirm the presence of atherosclerotic lesions in the area of elution, adjacent ring sections were monitored by histomorphometric trichrome staining (Fig. 2, b, d, f, and h) and by ex vivo high resolution NMR microscopy imaging (Fig. 2, a, c, e, and g). After eight months of cholesterol diet, the aorta showed complex plaque formation (Fig. 2, d, f, and h) with intimal thickening and macrophage infiltration in high shear stress regions that led to the formation of necrotic cores (Fig. 2, d and f, red arrows). After histochemical staining, macrophages have purple coloring while the collagen fibers have green coloring. Intramural thrombi (Fig. 2f) or blood decomposition products issuing from intraplaque hemorrhage (Fig. 2h) have developed afterangioplasty-induced injury and appeared, respectively, as red or black coloring in histology. A significant correlation between ex vivo NMR microscopy imaging and histological staining was observed concerning the visualization of plaque constituents. Different signal

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Intensities are given by distinct plaque components, *i.e.*, fibrous tissue, necrotic core, thrombi. Foam cells with high lipid content gave a gray signal on NMR microscopy images. In Fig. 2d, macrophage and collagen fibers are interspersed, leading to non-uniform coloring in the corresponding NMR microscopy image (Fig. 2c). They are closely packed, defining a high cellular density both in the subendothelial layer and deeper in the intima (Fig. 2, c and d). For the fibrous tissue with high collagen content, a brighter signal is detected (Fig. 2e). Intramural thrombi (Fig. 2, f and h, green arrow) and optically null regions such as necrotic cores (Fig. 2, d, f, and h, red and green arrows) appear as dark areas (Fig. 2e) or as a focal signal loss (Fig. 2, c and g). Ex vivo NMR microscopy imaging allows a fast and efficient confirmation of plaque composition before phage elution.

**Analysis of MoPhabs by ELISA**—To confirm the reactivity of colony filtered selected clones containing a full-length insert (800 bp), MoPhabs were screened using a conventional ELISA against atherosclerotic plaque proteins and those from normal aorta sections. As can be seen in Fig. 3, after one round of *in vivo* biopanning and selection by subtractive colony screening, ELISA reactivity of most of the selected MoPhabs was stronger using the atherosclerotic protein extract, especially for B2-7, B2-10, B2-11, B2-12, B3-GER, and B3-3G clones.

**Immunohistological Analysis**—After ELISA analysis, reactive MoPhabs were tested for binding specificity to early atherosclerotic lesions, to more complicated lesions obtained after balloon angioplasty in a rabbit model and to human carotid lesions. Sections of respective lesions were stained with Mason’s trichrome to show the morphology of the different plaques (Fig. 4, a–c). Deparaffinized sections of diseased and

![FIGURE 2. Localization of atherosclerotic plaques by NMR microscopy imaging (a, c, e, g) and histology (b, d, f, h). FLASH sequence parameters were described under “Experimental Procedures.” a and b, aorta of rabbits fed a normal diet. The medium is highly regular, and the intima is covered by a monolayer of endothelial cells. c and d, atherosclerotic lesions developed in the aorta of a rabbit fed a cholesterol-rich diet for eight months. The medium is highly disorganized and the intima extensively thickened. e–h, atherosclerotic lesions developed in the aorta of a rabbit fed a cholesterol-rich diet for eight months and subjected to two angioplasties (5 weeks before the diet and 1 week before the sacrifice). Intramural hemorrhages are triggered by the mechanical injury. Legend: red arrows designate the necrotic core; green arrows designate the intramural thrombi. Scale bar: 200 μm.](image-url)

![FIGURE 3. ELISA of MoPhabs selected by colony screening on proteins (50 μg/ml) from aorta with atherosclerotic plaques and from control aorta coated on a 96-well ELISA plate. Absorbances were measured at 405 nm.](image-url)
healthy aortas were incubated with $10^{10}$ pfu of MoPhabs or insertless phage as a control. Two MoPhabs called B3.3G (Fig. 4e) and B3.GER (Fig. 4f) showed similar and specific binding to the intimal surface of early atherosclerotic lesions. When the same number of insertless phages was applied on an adjacent section, no binding was detected (Fig. 4d). Anti-M13 antibody plus secondary antibodies have also served as negative controls (data not shown). B3.3G (Fig. 4g) and B3.GER MoPhabs (Fig. 4h) did not stain the intimal surface of a healthy rabbit aorta. Soluble scFvs B3.3G and B3.GER containing a c-Myc tag and a His$_6$ tag were next produced in small scale cultures, purified by immobilized metal affinity chromatography, and applied to deparaffinized sections of diseased regions of aorta. Binding was revealed by an anti-c-Myc mouse mAb and peroxidase-conjugated anti-mouse secondary antibody. Specific uptake on the intimal surface of early atherosclerotic plaques from rabbit aorta. On more complicated lesions, scFv B3.3G binding is observed within the plaque (k). Anti c-Myc antibody plus secondary antibody (l) or secondary antibody alone (m) were used as negative controls. Sections of human carotid atherosclerotic lesions were stained with soluble scFv B3.3G (o). Anti c-Myc antibody was used as negative control (n). Legend: 1, atherosclerotic intima thickening; 2, endothelial layer; 3, lumen; 4, media layer; 5, thrombus; 6, necrotic core.

In Vivo Localization of MoPhabs B3.3G in ApoE Mice—MoPhabs B3.3G were analyzed further for in vivo homing to atherosclerotic lesions developed in ApoE mice fed an atherogenic diet for several months. MoPhabs B3.3G were injected into mice via the tail vein and permitted to circulate for 30 min. After sacrifice of the animal, washing, and fixation of the entire aorta, the red-brown staining using anti-M13 peroxidase-con-
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B3.3G Binds to Vitronectin—To identify the B3.3G target antigen, we next performed immunoprecipitation experiments with soluble scFvs with c-Myc tag using proteins extracted from atherosclerotic aorta and normal aorta. After isolation of protein-bound scFvs using anti-c-Myc paramagnetic microbeads and analysis of the eluted products by SDS-PAGE, we have excised a band around ~56 kDa, which was not present for the controls (immunoprecipitation with normal aorta protein extract and with anti-c-Myc paramagnetic microbeads alone). This protein, which migrated at ~56-kDa, was subjected to trypsin digestion and sequenced by mass spectrometry as described under “Experimental Procedures.” Two peptides (FEDGILDPDYPYR and IYISGLTPSPSAK) showed exact identity with rabbit vitronectin (also known as serum spreading factor or GP66). ELISA confirmed the reactivity of B3.3G MoPhabs with purified human vitronectin (Fig. 6b).

DISCUSSION

Subtractive Filter Screening after Single Round in Vivo Biopanning—In this study, we have applied modern methods of antibody technology allowing the early isolation of human scFvs targeting atherosclerotic components in rabbits fed a fat-rich diet. We have used a phage display library expressing on their coat proteins human synthetic antibodies in the scFv format to probe atherosclerotic lesions in vivo. Endothelial cell dysfunction is a critical event in atherosclerosis and molecular changes at the endothelial surface result in the initiation, the progression, and eventually the pathologic complications of atherosclerosis. The specific signature of developing atherosclerosis is often hard to study by conventional methods since endothelial cells lose their integrity under such conditions. Phage display in vivo has shown to be the less biased approach and has been widely used to isolate peptide moieties targeting vasculature in several tissues and in several disease models including atherosclerosis (19, 20). A significant advantage of in vivo phage-display technology is simultaneous negative and positive screenings. Targeting of selectively expressed molecules within particular atherosclerotic regions but with no reaction with normal tissue represents an optimal selection process. Nevertheless, major drawbacks remain: 1) so far, the classical way to isolate specific ligands (peptides or rarely antibody fragments) for a particular tissue by in vivo phage display consists of repeated rounds of panning followed by random picking of individual clones and testing by ELISA or immunohistochemistry. This is a time-consuming strategy. 2) The multiplication of selection rounds decreases the complexity of the selected repertoire (11). This is due to a growth advantage of clones directed against abundant epitopes, particularly when the selection is performed against highly complex targets such as extracts of whole cells. 3) The time that phages have been allowed to circulate is often short and does not allow them to be incorporated deep within the forming plaque. Therefore, to isolate the maximum diversity of MoPhabs, we have chosen a 2-h MoPhab circulation period and we have analyzed clones after just one round of in vivo biopanning. Our strategy is based on a subtractive screening with a normal aorta protein-coated filter allowing the early selection of MoPhabs reactive with atherosclerotic components. Filter screening has been used previously to screen libraries against purified protein-coated filters (12, 21) or cell-coated filters (22). De Wildt et al. (11) have demonstrated the possibility to isolate scFv directed against diluted targets in a complex cell. This procedure that we have used offers many advantages compared with the classical method for isolating recombinant antibodies (several rounds of panning followed by random picking and flow cytometry or ELISA analyses). First, in vivo biopanning can be kept to a minimum (here one round of selection was applied for the recovery of low frequency and disease-specific clones). Second, more than 300 individual colonies can be screened simultaneously, and last, in situ expression avoids denaturation of antibodies that frequently occurs in more time-consuming assays. A major advantage of our technique compared with previously described filter methods is the screening of MoPhabs rather than soluble scFVs. Indeed, we have observed in our laboratory that MoPhabs are often more reactive than their soluble form. Moreover, specific selected VH-VL pairing can be easily transferred in cassette vectors for eukaryotic expression to obtain an antibody fragment (Fab or Fab’2) or a complete functional mAb more stable than a scFv obtained in E. coli (23). We have shown that high resolution ex vivo NMR microscopy is a fast and efficient tool for the deter-
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FIGURE 6. a, immunoblot experiment with B3.3G. Western blot of atherosclerotic aorta protein extract (lane 2) and normal aorta protein extract (lane 3) with B3.3G scFv (lane 1, marker; lane 3, anti-His tag alone on atherosclerotic protein extract). B3.3G revealed two distinct bands around 56 and 50 kDa. b, ELISA of B3.3G MoPhab on bovine serum albumin (50 μg/ml) or human vitronectin (5 μg/ml) coated on a 96-well ELISA plate. Absorbances were measured at 405 nm.

Conclusion and Perspectives—Our results demonstrate that a subtractive colony filter screening is a viable approach for isolating disease-specific human antibody fragments among a large panel of candidates after a single round of in vivo selection. This technique can be applied to any disease where a differential molecular profile exists between a healthy and pathologic tissue. Moreover, in vivo phage display is an emergent procedure for the isolation of scFvs that are selected on the basis of their in vivo behavior with criteria including stability, biodistribution, and reactivity with attractive targets. This faculty to target atherosclerotic lesions has been confirmed for B3.3G by in vivo homing on ApoE−/− mice that have a heavy thickening of the aortic intima. When coupled with contrast agents, B3.3G produced as scFv fragments in E. coli or as Fab or Fab’2 fragments in insect cells, are potentially very promising tools for use as non-invasive magnetic resonance imaging agents for the detection of a broad range of atherosclerotic lesions, thereby aiding the management of vulnerable patients. We will further evaluate their possible use in therapy. Indeed, it has recently been reported that local inhibition of vitronectin function prevents neointima formation after arterial injury (28). Targeting vitronectin may be an efficient way to limit plaque formation.

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mination of plaque complexity, which is an important prerequisite of the elution step. By combining in vivo phage display, ex vivo controlled MoPhab tissue elution, and MoPhab filter screening, we have succeeded in isolating MoPhabs binding at the surface or within the atherosclerotic plaque. Two of them, B3.3G and B3.GER, are interesting candidates for use as diagnostic tools or in therapy targeting. They recognize epitopes on the surface of the plaque in early stages of the disease and within the plaque in more complicated injuries. Importantly, B3.3G and B3.GER MoPhabs did not bind to the vasculature of a normal diet fed rabbit. Moreover, both antibodies are able to recognize conserved targets common to rabbit and human atherosclerotic disease as demonstrated by immunohistochemistry performed on dissected human arterial atherosclerotic lesions.

B3.3G Targets Vitronectin—B3.3G has been the object of extensive further examination. The data presented here indicate that the homing target of B3.3G is vitronectin. Vitronectin is a multifunctional glycoprotein, present in plasma, extracellular matrix, and in the alpha granules of platelets; it plays a role in the migration, the attachment, and the differentiation of cells (24). A number of reports (25, 26) indicate that vitronectin accumulates in atherosclerotic plaques and is strongly expressed after vascular injury. Immunohistological staining of B3.3G on the surface of early lesions and within the plaque, near the necrotic core, where lipids are deposited in more complex plaques, is in good agreement with previously published data (27). Immunoblot experiments have shown that B3.3G detected two bands at ~56 and ~50 kDa. Interestingly, Mori et al. (27) have previously characterized the 56- and 50-kDa bands as the forms of vitronectin markededly increased in atheromatous aorta in Watanabe heritable hyperlipidemic rabbit. Taken together, these results prove that vitronectin is an attractive target for imaging atherosclerotic lesions.
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