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Pharmacokinetic Properties of Bivalent Miniantibodies and Comparison to Other Immunoglobulin Forms

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ABSTRACT

The pharmacokinetics of bivalent miniantibodies were compared in the mouse, and compared with those of a single-chain Fv (scFv) fragment and a whole antibody. Miniantibodies are based on scFv-fragments, which are fused, via a flexible hinge-region, to association domains consisting of different kinds of amphipathic, associating helices. All molecules in this study had the same binding site for antigen, which was not a murine molecule, and thus the kinetics independent of localization phenomena could be measured. The miniantibodies were found to have plasma elimination half-lives somewhat slower than a scFv fragment but much faster than a whole antibody, to be almost quantitatively excreted (via the kidneys) and to show no sign of breakdown in the plasma. Since miniantibodies can easily be prepared from *Escherichia coli* and show a greatly increased surface binding compared to monovalent scFv fragments, they should be useful molecules for *in vivo* diagnostics and therapy.

INTRODUCTION

Single chain Fv fragments (scFv) represent potentially useful molecules for targeted delivery of drugs, toxins or radionuclides to tumor sites (1-7). In comparison to intact antibodies, several major advantages make scFv-fragments attractive for *in vivo* diagnostic and therapeutic uses: A lower immunogenicity should result from the lack of constant regions. The smaller size (24 kD as opposed to 150 kD of an intact MAb) should lead to a lower retention in non-target organs and a better tumor penetration (8,9). The latter property, however, is somewhat counteracted by the loss of bivalency. By having two binding sites, whole antibodies generally show higher functional affinity (avidity) to surface-bound or polymeric antigens. This effect can be understood as a consequence of local concentration effects because, after docking one antigen binding site of the antibody to the surface, the second antigen binding site is kept in the vicinity and therefore at very high local concentration (10,11).

To overcome this problem inherent to the scFv fragments, we have previously designed and characterized bivalent miniantibodies, which combine small size with bivalent binding (12,13). The miniantibody concept was an attempt to recreate the distance of the two binding sites from a whole antibody, yet with much smaller molecules which would assemble in *Escherichia coli*. While there are several ways to chemically or genetically couple scFv fragments (14,15), only the miniantibody design retains the original geometry of the two binding sites in the whole antibody, which may be assumed to have been optimized in evolution.

The two miniantibodies investigated with a dimer molecular weight of about 64,000 are based on scFv-fragments fused, via a flexible hinge-region, to association domains, which consist of different kinds of amphipathic,

associating helices (12,13). The modular design of the association domains provides a structural independence from the fused antibody fragments and leads to a targeted dimerization of the fragments *in vivo*. The scdHLX miniantibody is held together by the formation of a putative four-helix bundle (16,17), and the scZIP miniantibody by the formation of a parallel coiled coil helix, also termed leucine zipper (18,19). A schematic representation of the fragments used in this study is shown in Fig. 1, and more detailed models have been described previously (12,13).

Bivalent miniantibodies can be isolated in fully functional form in a one-step affinity purification without any further need of refolding or chemical coupling (12,13). In high cell density fermentation, expression yields of about 200 mg/l bivalent scdHLX miniantibody were obtained (13), indicating the availability of the large amounts required for clinical studies.

The bivalent miniantibodies should therefore combine the considerably higher functional affinity (avidity) and a small size comparable to an Fab fragment. Our initial experiments were carried out with a murine antibody not directed against a murine antigen, in order to distinguish the pharmacokinetics independent of any localization phenomena. We used the well-characterized IgA antibody McPC603, which binds to phosphorylcholine (20). The scFv fragment (in the orientation V_H-linker-V_L) (21) and the two miniantibodies scZIP and scdHLX (12,13) thus share the same binding site. The recombinant molecules were produced in *Escherichia coli.*

The knowledge of the pharmacokinetic properties of a miniantibody compared to intact Ig and scFv is very important as a base for further investigations. The plasma kinetics will be decisive for judging the suitability of the miniantibodies for imaging (requiring a rather fast clearance rate) or therapeutic applications, which require a sufficient residence time in the body.

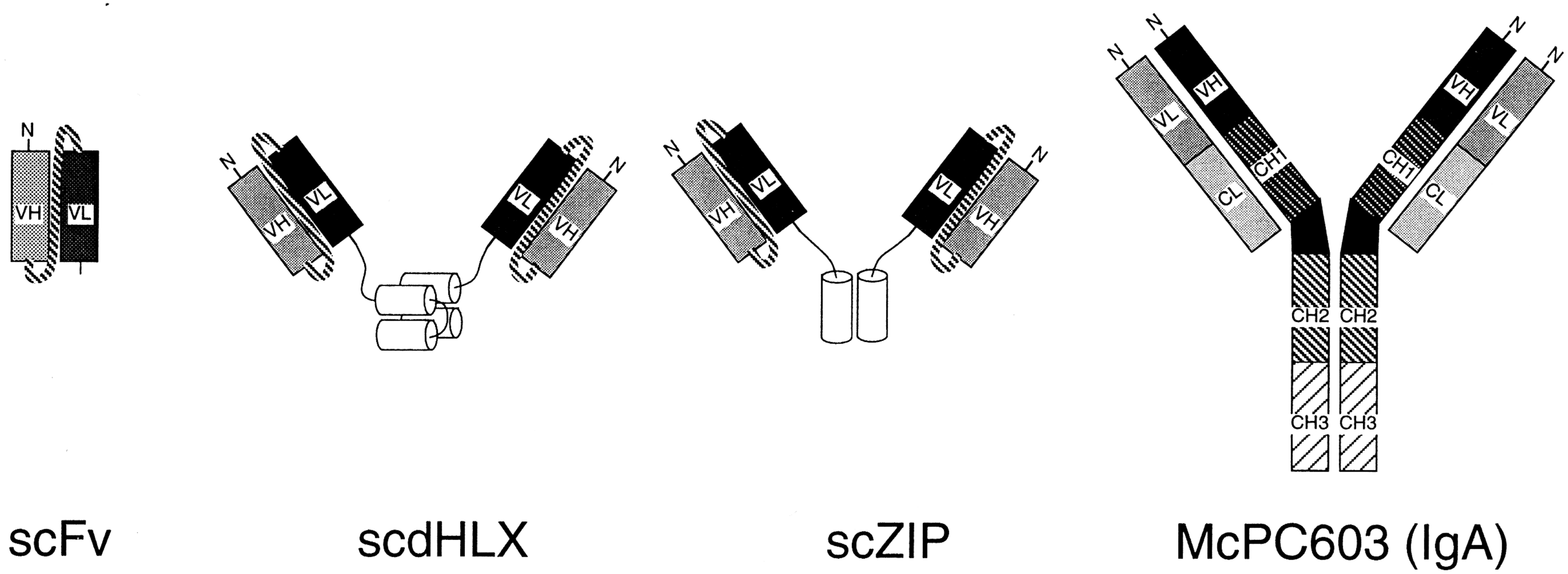

Figure 1: Schematic representation of the antibody fragments used in this study. All antibody fragments have identical variable domains. McPC603 is an IgA, which was purified in the H_2L_2 form.

The aim of the study in mice was first to compare the *in vivo* pharmacokinetic properties of an intact MAb with two miniantibodies and one scFv-fragment differing predominantly in their molecular size, second to investigate and compare the *in vivo* stability of the different miniantibody constructs and third to investigate the excretion behavior of the new entities. All four antibody-like molecules were iodine-labeled and the plasma kinetics and excretion, as well as the possible proteolytic degradation were investigated.

MATERIALS AND METHODS

Antibody and fragments (Proteins)

The construction, expression and purification of the scFv fragments and miniantibodies from *Escherichia coli* has been described previously (12,13). Briefly, for these experiments *E. coli* JM83 transformed with the appropriate plasmid (12,13) was grown in shake flasks in LB medium at room temperature, the cells were opened by French Press lysis and the functional recombinant antibody protein was purified by phosphorylcholine affinity chromatography. The IgA McPC603 was obtained from myeloma, mildly reduced to obtain the H_2L_2 form, and the protein was then affinity purified on phosphorylcholine-Sepharose as described (22,23).

Radioiodination

Proteins were labeled with ^{125}I using Iodogen-coated glass vessels prepared by standard methods (24). Two hundred μg protein and 400 μCi (14.8MBq) ^{125}I in 10 μl of water were added; after 10 minutes of incubation, the solution was removed from the vessel to stop the reaction, and the labeled protein was separated from the unreacted iodine by HPLC-gel filtration using

a Sorbax GF 250 column (DuPont/Dreieich). The specific activities thus obtained were in the range of 1.1 to 1.6 µCi/µg (0.04 to 0.06 MBq/µg).

Animals

All studies were carried out in six- to eight-week-old B6C3F1 mice weighing 25 to 30 g which were obtained from Charles River/Sulzfeld. The animals were housed in polycarbonate cages on a softwood granulate bedding (Altromin, Lage/Lippe).

The uptake of iodine into the thyroid was blocked by adding 40 drops/l of a perchlorate solution (Irenate®) to the drinking water of the animals four days prior to and during all days of the study.

Pharmacokinetics

Six mice per group (3 f and 3 m) were injected intravenously with 2.5 µCi each of ^{125}I-labeled MAb, scFv or miniantibodies. Blood samples of about 40 µl were taken under light ether narcosis from the retroorbital venous plexus using a heparinized glass cannula. After centrifugation for 2 min at 1000 rpm, 10 µl of the supernatant plasma was measured in a gamma counter (LKB Wallace).

The results are given in µg equivalent/ml (µg eq/ml), taking into account that determination of radioactivity includes both undegraded proteins and their labeled degradation products. The kinetics were fitted to a two-exponential decay and expressed as the half-lives of both phases.

Excretion with urine and feces

To determine the excretion of radioactivity into urine and feces the animals were kept in special metabolism cages (WOETHO/ Emmendingen).

The collection intervals were 0-8 and 8-24 h for scFv, scZIP, and scdHLX, and 0-8 h followed by 24 h-intervals up to 120 h for the whole IgA McPC603. Aliquots of the urine were measured directly, while the feces were homogenized prior to measurements of radioactivity. The remaining radioactivity in the carcass was determined after completion of the excretion study.

SDS PAGE and autoradiography

Plasma samples were subjected to SDS gel electrophoresis (with and without prior reduction by 2-mercaptoethanol) on 8 to 18 % poly-acrylamide gradient gels (25), and the radioactivity was localized by autoradiography on X-ray film (Osray M3, Agfa-Gevaert).

RESULTS

Radiolabeled proteins

After radiolabeling the various proteins, the integrities of the MAb (IgGA), the two recombinant miniantibodies (scZIP and scdHLX) and the single chain fragment (scFv) were analyzed by SDS-PAGE followed by autoradiography. Under reducing conditions, it was found that mainly the heavy chains of the IgA were radiolabeled. The single protein chains of scFv, scZIP and scdHLX could be labeled to comparable specific activities (Fig. 2).

Pharmacokinetics

The pharmacokinetics of the proteins were evaluated by measuring the radioactive content of plasma samples at selected time points. As can be seen in Fig. 3, the plasma concentration for all four proteins decayed biphasically. Both the scFv fragment and the miniantibodies, scZIP and scdHLX, were

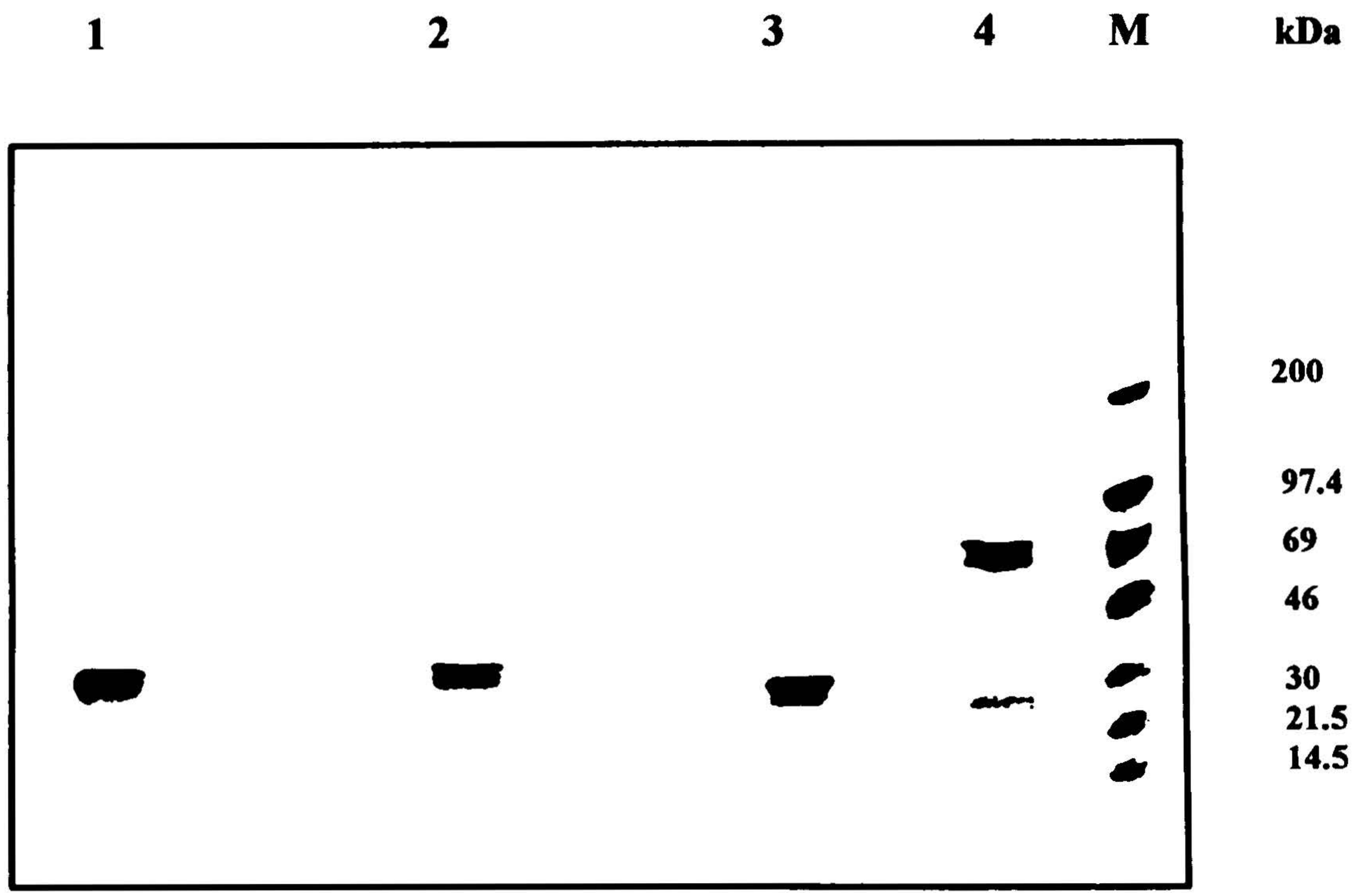

Figure 2: Autoradiography of ^{125}I-labeled MAb and MAb-fragments. Lane M, protein size marker (14.5; 21.5; 30; 46; 69; 97.4 and 200 kDa) lane 1, scZIP; lane 2, scdHLX; lane 3, scFv; lane 4, McPC603.

cleared more rapidly from the plasma than the whole IgA McPC603. Identical time intervals were used for calculating the α-phase (0-15 min) and β-phase (1-7 h) of the plasma elimination half-lives of the scFv fragment and the miniantibodies, scZIP and scdHLX. The initial distribution phase $t_{1/2}$ α for the scFv was 8.1 min and the elimination phase $t_{1/2}$ β was about 2.8 h (Table I). For the scdHLX and the scZIP the resulting α-phases were calculated to be 17.5 min and 11.9 min, and the corresponding β-phases were 3.4 h and 4.1 h, respectively. The half-lives of MAb McPC603 were 1.4 h (time interval: 0-2 h) and 21.1 h (time interval 4-120 h) respectively.

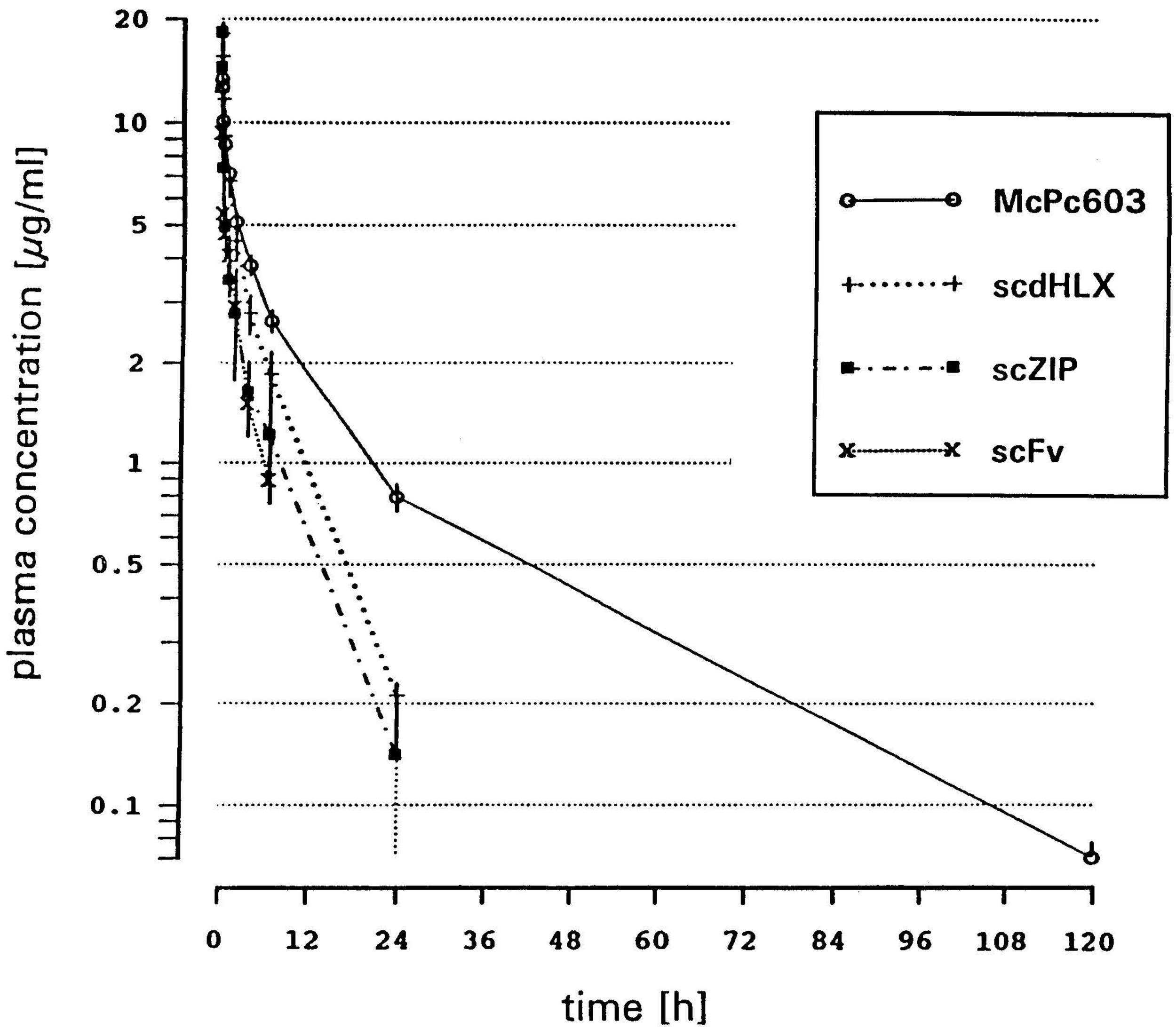

Figure 3: Time courses of ^{125}I-plasma concentration of MAb and MAb-fragments. Mice were given iv injections of 2.5 µCi of ^{125}I-McPC603, ^{125}I-scZIP, ^{125}I-scdHLX and ^{125}I-scFv. Blood samples were obtained at the times indicated and the ^{125}I activity was measured in a gamma counter. Values shown are means (n= 6) +/- SD.

Table I: Half-lives of MAb McPC603 and MAb-fragments

MAb/Mab fragments	$t_{1/2}$ α (min)[a]	$t_{1/2}$ β (h)[a]
scFv*	8.1 ± 2.6	2.8 ± 0.3
scdHLX*	17.5 ± 3.8	3.4 ± 0.3
scZIP*	11.9 ± 4.3	4.1 ± 1.3
McPC603+	84.0 ± 7.3	21.1 ± 0.7

[a]Mean ± SD

mice (n=6)were given intravenous injections of 2.5 μg of ^{125}I-Mab or MAb-fragments

* $t_{1/2}$ α (time interval: 0-15 min) and $t_{1/2}$ β (time interval: 1-7 h)

+ $t_{1/2}$ α (time interval: 0-120 min) and $t_{1/2}$ β (time interval: 4-120 h)

Excretion with urine and feces

The total percentage of radioactivity excreted with urine and feces is shown in Table II. Within 24 h the radioactivity in urine ranged between 69% and 82% for scdHLX, scZIP and scFv. Up to a maximum of 12% of the radioactivity was excreted into feces, amounting to a total excretion of 81% to 89% already after 24 h. In contrast, the whole IgA McPC603 was eliminated much more slowly (61% of total excretion within 24 h). Within 120 h a total of 70% was recovered in the urine and 12.3% in the feces. These results show that the small proteins are excreted more rapidly than the whole antibody, the main route being via the kidneys with fecal excretion playing only a minor role.

Determination of intact protein in the plasma

To confirm that the radioactivity in the plasma samples represented intact scZIP, the plasma samples were run on SDS-PAGE gradient gels, followed by autoradiography (Fig. 4). In this figure it is shown that the only visible radioactive bands in the plasma migrated in accordance with the molecular mass of intact ^{125}I-scZIP, demonstrating that the protein is stable in plasma and no degradation had taken place. The time course of disappearance of

Table II: Comparison of excretion of ^{125}I-radioactivity (% of dose) into urine and feces

Collection period		McPC603[a]	scdHLX	scZIP	scFv
[h]		mean	mean	mean	mean
	urine	41.4	35.1	62.7	49.1
0 - 8	feces	1.8	3.6	2.0	1.7
	total	43.2	38.7	64.7	50.8
	urine	52.5	68.6	82.4	79.0
0 - 24	feces	8.5	12.4	6.3	6.5
	total	61.0	81.0	88.7	88.5
	carcass[b]	-	8.7	10.8	7.8

[a] Collection period 0 - 120 h: urine 70.1% of dose and feces 12.3% of dose (total 82.4% of dose);
carcass after 120 h: 1.83% of dose.

[b] residual radioactivity in the animal body

Mice (n = 6) were given iv injections of 2.5μCi of ^{125}I-MAb or MAb-fragments.

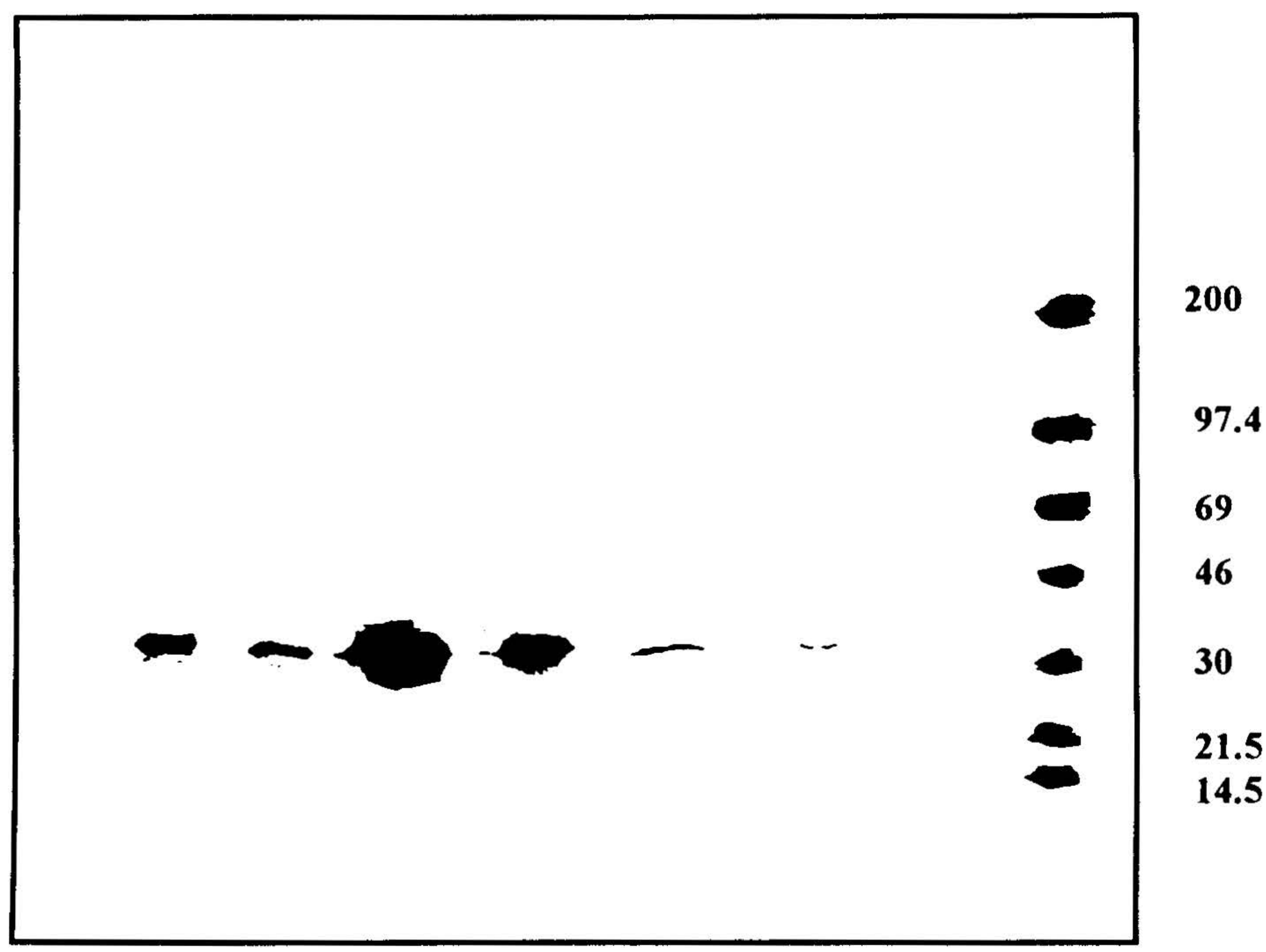

Figure 4: Stability of ^{125}I-scZIP in mouse plasma.
Lane F of the autoradiography is ^{125}I-scZIP spiked in normal plasma; lane 1-4, plasma at 5 min, 1 h, 7 h and 24 h postinfusion; lane M, protein size marker (14.5; 21.5; 30; 46; 69; 97.4 and 200 kDa).

^{125}I-scZIP from plasma follows the appearance of radioactivity in urine, indicating that the iodine label can be used as a valid method to determine the pharmacokinetics of the antibody molecules.

DISCUSSION

Studies were carried out to provide a first set of data on the plasma clearance of bivalent radiolabeled miniantibodies (scdHLX and scZIP) in mice and to compare them to the plasma clearance of radiolabeled scFv and intact IgA all containing the same antigen binding site as the antibody McPC603 (20). For this purpose, the murine model antibody McPC603 (20) and its

recombinant forms, scFv, scdHLX and scZIP, all three expressed in *E. coli*, were used. As this model antibody does not bind to mouse tissue, exclusively the pharmacokinetic properties can be observed. The results demonstrate that plasma clearance rates are decreasing in the order of the molecular weight.

Interestingly, the *in vivo* pharmacokinetics of the miniantibodies scdHLX ($t_{1/2}$ of 17.5 min for the α-phase and 3.4 h for the β-phase) and scZIP ($t_{1/2}$ of 11.9 min for the α-phase and 4.1 h for the β-phase) resulted in half-lives comparable to that of Fab molecules, such as CC49 Fab ($t_{1/2}$ of 9.1 min for the α-phase and $t_{1/2}$ of 1.5 h for the β-phase) and B6.2 Fab ($t_{1/2}$ of 14.8 min for the α-phase and $t_{1/2}$ of 7.5 h for the β-phase) in tumor bearing nude mice (5,6). Thus, the plasma clearance of proteins with comparable overall molecular weight (Fab-fragments and miniantibodies) seems to be similar, despite the different molecular shape, and the presumed higher flexibility within the bivalent miniantibody.

The half-life of McPC603 scFv is consistent with those of previously examined scFvs, for example CC49 and B6.2, determined in tumor bearing nude mice. Their plasma half-lives were shown to be 2.4 min (α-phase) and 2.8 h (β-phase) for the B6.2 scFv, 3.7 min (α-phase) and 1.5 h (β-phase) for the CC49 scFv (5,6) and 8.1 min (α-phase) and 2.8 h (β-phase) for the McPC603 scFv. Furthermore, the plasma clearance of scFv in mice was comparable to that observed in rhesus monkeys as reported for CC49 scFv ($t_{1/2}$ of 3.9 min and $t_{1/2}$ of 4.2 h) (6).

The rapid clearance of scFv, scdHLX and scZIP in mice is also reflected by the excretion studies, showing that scFv and both miniantibodies are excreted more rapidly than intact IgA (McPC603). Within 24 hours, about 89% of the dose are eliminated from the body in the case of the McPC603 scFv. Similar results were obtained for the CC49 scFv of which more than 90% were

excreted by xenografted mice within 24 hours (6). Whole body clearance of B6.2 Fab demonstrated that more than 60% of the injected dose was eliminated after 6 h (5). Thus, after 8 h the degree of elimination of scdHLX and scZIP is on the same order of magnitude (40% and 65%, respectively, Table II). At the moment no information about the significance of the differences in the excretion rate during the first 8 h period between the two miniantibodies is available.

These excretion and the carcass data indicate that scFv and miniantibodies are rapidly and quantitatively eliminated from the body and that obviously no major retention occurs in any organs or tissues. This result is therefore an important prerequisite for any medical applications of these new molecules.

The ability of B6.2 scFv to target a human tumor xenograft in mice was shown to be similar to that of Fab fragments. Tumor to normal tissue ratios of B6.2 scFv resulted in comparable or slightly greater ratios than those obtained with Fab fragments. Thus, it is suggested that because of the similar size of Fab-fragments and miniantibodies, a comparable uptake into tumors might occur, although miniantibodies should be more advantageous due to their bivalent binding ability.

Furthermore, a number of studies performed in mice and humans have shown that Fab fragments accumulate in the kidneys to much higher levels than do scFv proteins (5, 26-28). Whether miniantibodies show similar unwanted accumulation has yet to be examined.

Above all, there are some potential advantages which could make miniantibodies more useful for tumor therapy than scFv and intact Ig: They are recombinant proteins that can be expressed in *E. coli* and, therefore, cannot

be contaminated by any hazardous products derived from eukaryotic cells, and they may be less immunogenic in patients because all domains except the V_H and V_L are deleted. Furthermore, being recombinant molecules, a humanized (29) version would be just as easily produced. Moreover, they combine small size with high binding avidity and rapid clearance, and might therefore be of interest in imaging, but also in therapeutic applications. Finally, the general dimerization devices may be used to couple other molecules to antibodies, with only minimal size requirements, and compatible to large scale production in bacteria.

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