Pharmacokinetics and Organ Distribution of Cationized Colchicine-Specific IgG and Fab Fragments in Rat

GUIYING HONG,* MARTINE I. BAZIN-REDUREAU, AND JEAN MICHEL G. SCHERRMANN

Contribution from *Inserm U26 and Department of Pharmacokinetics, University of Paris V, Hôpital Fernand Widal, Paris, France*

Received August 21, 1997. Final revised manuscript received June 10, 1998.
Accepted for publication September 2, 1998.

Abstract □ Pharmacokinetics of cationized goat colchicine-specific polyclonal immunoglobulin G (IgG) and antigen binding fragment (Fab) (cIgG and cFab, respectively) were studied in male adult Sprague–Dawley rats and compared with those of the native proteins (nIgG and nFab). All proteins were radioiodinated by the Iodogen method, and kinetics were investigated following trichloroacetic acid (TCA) precipitation or immunoprecipitation. Deiodination and catabolism were more pronounced with the cationized than the native proteins, especially for cFab. Both cIgG and cFab in plasma decreased more rapidly than nIgG and nFab. The elimination half-lives were 52.9 and 81.8 h for cIgG and nIgG, respectively. In addition, there was a 74-fold increase in the volume of distribution and a 114-fold increase in the systemic clearance of cIgG compared with nIgG. For cFab, the volume of distribution and systemic clearance were increased 6.4- and 3.5-fold, respectively. Organ uptake of cIgG and cFab was markedly increased compared with that of nIgG and nFab, especially in kidney, liver, spleen, and lung. Renal clearance of cIgG and cFab was also increased 30- and 10-fold compared with that of nIgG and nFab, respectively. The present data suggest that cationization of colchicine-specific IgG and Fab fragments increased the organ distribution and greatly altered their pharmacokinetics. Nevertheless, the smaller molecular size of Fab versus IgG did not enhance the distribution and clearance of cFab. These data pave the way for evaluating the biological efficacy of these more tissue-organ-interactive antibodies.

Introduction

Antibodies offer considerable potential for disease diagnosis and for treatments, such as the neutralization of bacteria, viruses, oncogenic proteins,[1] and toxins.[2] However, the diagnostic and pharmaceutical potential of antibodies is limited because these high molecular weight proteins [immunoglobulin G (IgG), 150 kDa] do not cross either capillary or cellular barriers.[3,4] Even trunctated forms of antibodies, such as antigen binding fragments (Fab, 50 kDa) or even sFv fragments (27 kDa), are too large to easily penetrate physiological membranes. Therefore, it is important to develop strategies for improving the delivery of antibodies to the site of action.

One of the recent developments in targeted delivery of proteins is their cationization,[5,6] in which the surface carboxyl groups of the protein are conjugated with primary amino groups resulting in an increase in the protein isoelectric point (pI). The positive charges of the cationized proteins so formed bind to negative charges on cellular surfaces and thus trigger absorptive-mediated endocytosis of the cationized proteins.[7] This interaction between cell membrane and cationized proteins can be evaluated by pharmacokinetic studies.

The first studies[5,8] concerning the pharmacokinetics of cationized IgG (cIgG) reported an enhanced organ uptake. However, the experimental protocols had a short sampling period and took little account of the influence of the different antibody size between cIgG and cationized Fab (cFab) fragments. In the present study, we report the plasma pharmacokinetics of cationized goat polyclonal colchicine-specific antibodies (IgG and Fab) and their organ distribution in the rat. In addition, their renal clearance and fecal elimination were also measured. This pilot study was conducted prior to assessing the potency of cationized colchicine-specific IgG and Fab for redistributing intracellular colchicine, a compound toxic to tubulin that either inhibits microtubule-dependent processes or disrupts the cytoskeleton.[9]

Materials and Methods

Materials—Goat IgG and Fab fragments of colchicine-specific polyclonal antibody were prepared as previously described.[10] IgG and Fab fragments were purified by gel chromatography to 97% and 92% for IgG and Fab fragments, respectively. Colchicine and thiocolchicine were obtained from Roussel Uclaf (Paris, France) and [^{3}H]colchicine (Ring C,^{3}H-methoxy, 66 Ci/mmol) was from New England Nuclear (Paris, France). Hexamethylenediamine (HMD), *N*-ethyl-*N*′-(3 (dimethylamino) propyl)carbodiimide (EDC), glycine, and others reagents for protein cationization were obtained from Sigma (St Quentin Fallavier, France). Affinity purified rabbit anti-goat IgG (H and L) antiserum (Rockland, PA) and protein A-agarose specific to rabbit polyclonal antibodies (Santa Cruz Biotechnology, Inc.) were purchased from Tebu (Paris, France).

Cationization of Colchicine Specific IgG and Fab—Native colchicine-specific IgG and Fab were cationized according to the method of Pardridge[11] with a small modification: 10 mg of both native IgG and Fab were slowly added to 4 mL of 2 M hexamethylenediamine (HMD, pH 6.2). To this mixture, 50 mg of fresh EDC was added, and the pH was adjusted to 6.2 with 6 M hydrochloric acid. The mixture was stirred for 3 h at room temperature, and the solution was kept on ice to avoid heating. The reaction was quenched by addition of 2 M glycine following by incubation for 60 min at room temperature. The mixture was then dialyzed overnight at 4 °C against 0.01 M Na_2HPO_4/0.15 M NaCl (PBS, pH 7.4). The precipitate was removed by centrifugation (1000 g, 10 min) and the solution was concentrated with polyethylene (6000 Da) at 4 °C. The solution was stored at −20 °C.

Characterization of Cationized IgG and Fab—The pI of cationized and native IgG and Fab were determined by isoelectric focusing (IEF).[6] Protein samples (30 μ) were dialyzed with 0.05% glycine overnight at 4 °C to eliminate excess ions and analyzed by isoelectric focusing (IEF) according to the manufacturer's instructions (Sigma, St Quentin Fallavier, France). After isoelectrophoresis, the gel was stained with Coomassie blue. The cationized antibodies were also subjected to sodium dodecyl

* Correspondence should be addressed to Guiying Hong at INSERM U26, Hôpital Fernand Widal, 200, Rue du Faubourg Saint-Denis, 75475 Paris, France. Tel: 33 01 40 05 43 46. Fax: 33 01 40 34 40 64.

10.1021/js970335n CCC: $18.00
Published on Web 12/12/1998

sulfate−polyacrylamide gel electrophoresis (SDS−PAGE) analysis in 8% or 12% acrylamide, using the Laemmli buffer system, to determine the molecular weight of the cationized and native antibodies.[12]

The affinity constant was determined by competitive radioimmunoassay (RIA) according to the method of Müller.[13] Specificity of the native and cationized antibodies was measured by the cross-reactivity of a colchicine analogue, thiocolchicine, using the RIA as previously described by Scherrmann et al.[2]

Radiolabeling of IgG and Fab—Antibodies were labeled using the Iodogen method.[14] One hundred micrograms of protein were incubated with 0.5 mCi of [^{125}I]Na (Amersham, Les Ulis, France) in Eppendorf tubes coated with 10 μg of Iodogen for 5 min at room temperature. Free iodine was removed by chromatography on a PD-10 Sephadex G-25 column (Pharmacia, Les Ulis, France). Specific activity ranged from 2 to 3 μCi/μg for native IgG (nIgG), 1−2 μCi/μg for cIgG, 1−2 μCi/μg for native Fab (nFab), and 0.6−1.5 μCi/μg for cFab. The radiochemical purity of cIgG, nIgG, cFab, and nFab was analyzed just after iodination and 24, 48, and 72 h and once a week up to 3 weeks after the iodination by SDS−PAGE autoradiography and immunoprecipitation (for procedure see: Comparison of Plasma Antibody Levels following TCA Precipitation and Immunoprecipitation). Finally, the percentage of antibody precipitable radioactivity was determined by trichloroacetic acid (TCA) precipitation.

Intravenous Administration of Cationized and Native IgG and Fab Fragments—Male Sprague−Dawley rats (250−300 g, Iffa Credo, Lyon, France) were given free access to food and water. Each rat was weighed and anesthetized with pentobarbital sodium (60 mg/mL, 60 mg/kg, ip). Body temperature was monitored using a rectal probe (Elba Thermometer model ET 3, Copenhagen, Denmark). During the experiment, each rat was housed in a metabolic cage for collection of urine and feces at the interval of 8 or 16 h. The femoral vein was cannulated with PE−10 tubing (Biotrol, Paris, France). Rats received a single bolus dose of 0.7 mg/kg of unlabeled antibody and 15 μCi of ^{125}I-labeled antibody dissolved in 0.9% NaCl via the cannulated femoral vein. Blood samples were collected in heparinized tubes from the tail at various times (0, 2, 15, 30, and 60 min, 2, 4, 8, and 24 h, and daily for 18 days for nIgG; 0, 1, 10, 20, and 30 min, and 1, 2, 4, 6, 8, 24, 30, 48, 54, 72, 96, and 120 h for cIgG; and 0, 1, 10, 20, and 30 min, and 1, 2, 4, 6, 8, 24, 28, 32, 48, 56, and 72 h for cFab and nFab). Aliquots of plasma were counted for total and TCA-precipitable radioactivity in a gamma counter (Minaxi gamma 5000, Packard Instruments, Rungis, France). Other aliquots were stored at −20 °C for SDS−PAGE followed by autoradiography. Urine and feces were collected throughout the experimental period for measurement of total excreted radioactivity. Free ^{125}I in urine was determined by precipitation with TCA.

Comparison of Plasma Antibody Levels following TCA Precipitation and Immunoprecipitation—To check whether the TCA-precipitable radioactivity corresponded to the intact native or cationized proteins, an immunoprecipitation methodology was applied in a satellite group of rats ($n = 3$). Blood samples (1 mL) were taken 0 and 5 min, and 2, 8, 24, and 48 h for cIgG and at nIgG; and 0 and 5 min, and 2, 4, 8, 24, and 48 h for cFab and nFab after intravenous administration, respectively. Two aliquots of plasma samples were counted for total and TCA-precipitable radioactivity in the gamma counter. Immunoprecipitation was performed as follows: to 200 μL of each plasma sample (or 10 μL of pure radiolabeled native or cationized IgG and Fab diluted in 200 μL rat plasma for the radiochemical purity determination), 100 μL of incubation buffer was added and the solution was incubated at 37 °C for 3 h, then at 4 °C for 1 h. The incubation buffer consisted of PBS (10 mM sodium phosphate, pH 7.4, with 0.14 M NaCl) and 30 μg/mL of rabbit polyclonal anti-goat IgG. Protein A-agarose (40 μL) was added to each tube. After overnight incubation at 4 °C, the immunoprecipitate was collected by centrifugation at 5000 rpm for 7 min at 4 °C, and the supernatant was aspirated and discarded carefully. The immunoprecipitate was washed with PBS (300 μL) and centrifuged as before. This step was repeated three times. After final washing, the immunoprecipitate fractions were counted for radioactivity. The nonspecific precipitation evaluated using normal rabbit plasma was <1%.

Distribution of Cationized and Native Antibodies in Organs—Distribution of antibodies in organs was studied at different times: 6, 72, 120, and 192 h for cIgG; 6, 72, 432, and 456 h for nIgG; and 2 h for both cFab and nFab. Kidneys, liver, spleen, heart, lung, brain, thyroid, and a portion of small intestine were removed, rapidly washed with saline, and blotted dry. Fragments of organs were weighed and solubilized in Soluene 50 according to Pardridge et al.[15] at 60 °C for 60 min and counted for radioactivity in gamma scintillation liquid. Distribution of antibodies in the organ was expressed as ng/g of tissue. Blood samples were taken just before rats were killed, and radioactivity was measured. As radioactivity remaining in the blood at the time of death was very different for native and cationized antibodies, total radioactivity in the organs was corrected according to levels found in the blood.[16]

SDS−PAGE and Autoradiography—Plasma (0.1 mL) containing native or cationized ^{125}I-labeled antibodies was analyzed by SDS−PAGE.[12] Radiolabeled antibodies and metabolites were autoradiographed using X-ray film (Amersham) and intensifying screens for several weeks. The migration zone of native ^{125}I-labeled antibodies or fragments was compared with protein markers of known molecular weights (Rainbow, Amersham, France); they are, myosin (205 kDa), β-galactosidase (116.5 kDa), bovine serum albumin (80 kDa), ovalbumin (49.5 kDa), and soybean trypsin inhibitor (27.5 kDa).

Detection of Rat Anti-goat Immune Response to the Cationized and Native IgG and Fab—An immunoradiometric assay (IRMA) was performed to detect anti-goat cIgG, nIgG, cFab, and nFab in rat plasma 1, 2, and 3 weeks after administration of a single dose (700 μg/kg) of each antibody. Microtitration plates (Falcon microtest III U50, Polylabo, Strasbourg, France) were coated with 200 μL of 100 mM sodium carbonate pH 9.6 containing 20 μg/mL of affinity-purified goat nIgG, nFab, cIgG, and cFab, respectively, by overnight incubation at 4 °C. The plates were washed three times with PBS containing 0.2% Tween-20 (PBS-Tween). Overcoating was achieved by a 1-h incubation at room temperature with 200 μL/well PBS containing 3% BSA to reduce nonspecific serum protein binding. Plates were then washed three times with PBS-Tween. Then, 100 μL of rat plasma samples, 100 μL of normal rat plasma containing 1 μg of rabbit anti-goat IgG (positive control), or 100 μL of normal rat plasma (nonspecific binding) were added to the sensitized plates and incubated for 2 h at room temperature. All assays were performed in triplicate. The plates were washed and incubated 1 h at room temperature with 100 μL of appropriate optimally diluted conjugate ([^{125}I]cIgG, [^{125}I]nIgG, [^{125}I]cFab, or [^{125}I]nFab, 70 000 cpm, respectively). Plates were washed three times with PBS-Tween and dried. Each well was cut out and placed in a 5-mL plastic tube. All tubes were capped and counted in a gamma counter. The radioactivity in each tube was expressed as % binding; that is, $(B - NS)/(T - NS) \times 100\%$, where B = bound, T = total radioactivity and N = the nonspecific binding of the tracer.

Pharmacokinetic and Statistical Analysis—Plasma cIgG, nIgG, cFab, and nFab TCA-precipitable concentrations were analyzed by model-independent techniques using the SIPHAR program (SIMED, Creteil, France). The terminal disposition rate constant (λ_d) was determined by linear regression analysis and the corresponding half-life ($t_{1/2}\lambda_d$) was calculated as $0.693/\lambda$. The area under the curve (AUC) of plasma ^{125}I-cationized or native IgG and Fab concentration versus time curve from zero to infinity was determined by linear trapezoidal estimation from zero to the last measured time with extrapolation to infinity by adding the value of the last measured plasma concentration divided by the terminal rate constant. Total body clearance (CL) and volume of distribution (Vd) were calculated using standard equations.[17]

Renal clearance was calculated by dividing the amount of protein in urine by the plasma AUC at the same time interval. Mean values for pharmacokinetic parameters were compared using one-way analysis of variance (ANOVA) followed by the Student-Newman-Keuls Multiple Comparisons Test (GraphPad software V 2.04 a). Significance was set at $p < 0.05$.

Results

Characteristics of Antibodies after Cationization and ^{125}I Radiolabeling—As measured by IEF, the pI of nIgG and nFab ranged from 5.85 to 9.0 and 8.65 to 9.3, respectively. The pI of cIgG and cFab were both raised and ranged from 8.65 to 10.3 and 9.5 to 11, respectively.

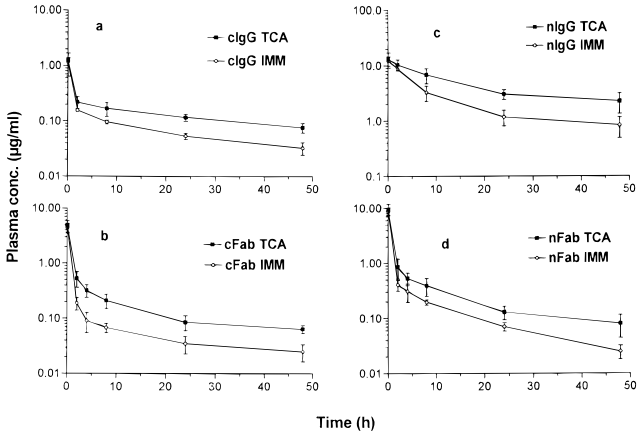

Figure 1—Time courses of plasma ^{125}I-labeled antibody concentrations precipitated by TCA and immunoprecipitation (IMM) in a satellite rat group. Graphs a, b, c, and d represent different antibodies. Data are mean ± SD (n = 3).

However, the process of cationization did not significantly change the affinity and specificity of IgG and Fab fragments. The affinity constants of cIgG and nIgG were 1.2 ± 0.13× 10^9 and 1.58 ± 0.2 × 10^9 M^{-1} (p > 0.05) respectively, and 1.08 ± 0.21 × 10^9 and 0.64 ± 0.18 × 10^9 M^{-1} (p > 0.05) for cFab and nFab, respectively. The cross-reactivity to thiocolchicine did not differ significantly between cationized and native forms. The percentages were 8.6 ± 1.13 and 8.9 ± 0.58% (p > 0.05) for cIgG and nIgG, respectively, and 25 ± 5.9 and 37 ± 8.67% (p > 0.05) for cFab and nFab, respectively. Furthermore, after cationization of IgG and Fab, high molecular weight aggregates were not detected by SDS−PAGE, and no alteration of the migration bands of the native and cationized antibodies were observed by autoradiography after iodination. Most (90−94%) of the total radioactivity of cationized and native antibodies was recovered by immunoprecipitation just after iodination or even 3 weeks later. The percentage of TCA-precipitable radioactivity of the native and cationized antibodies changed from 96−98% following iodination to 85 ± 5% at two weeks later. As a consequence, all pharmacokinetic studies were performed within 24 h after the radiolabeling of the native and cationized antibodies. Finally, we found that the cationized molecules bound nonspecifically to silicon and plastic tubing (nonspecific binding was 1.9 and 2.1%, respectively), we thus used glass tubing to avoid nonspecific binding (<1%).

Detection of Rat Anti-goat Immune Response to Cationized and Native IgG and Fab—The binding capacity of each antibody-treated (cIgG, cFab, nIgG, and nFab) rat plasma sample to its corresponding antigen accounted for <0.3% (but 46.12 ± 1.3% for the positive control) of the total radioactivity during the 3 weeks following the protein administration. This result indicates that no anti-goat immune response occurred during the experimental time period of the pharmacokinetic study after a single iv dose (700 µg/kg,) of the antibodies, not even for cationized IgG and Fab.

Comparison of Pharmacokinetics Following TCA Precipitation and Immunoprecipitation—Figure 1 shows the plasma concentration of cationized and native antibodies detected by TCA precipitation and immunoprecipitation in the satellite group of rats. Except at the earlier time of 5 min, the plasma concentration of each antibody detected by immunoprecipitation was lower than that detected by TCA precipitation, but plasma decline was parallel during terminal decay with both analytical methods. The ratios between immunoprecipitated and the TCA-precipitable AUC_{0-48h} were 0.53, 0.56, 0.41, and 0.39 for nIgG, cIgG, nFab, and cFab, respectively.

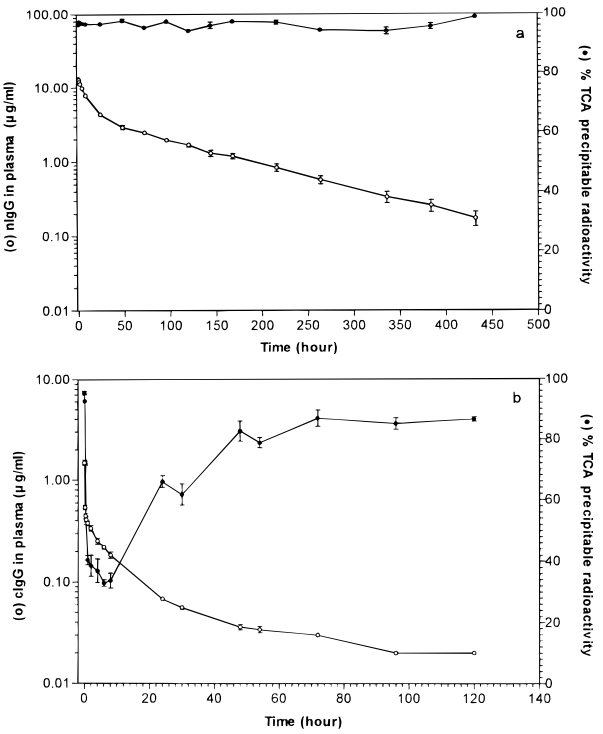

Figure 2—Plasma concentration of ^{125}I-nIgG and ^{125}I-cIgG. Plasma data were corrected for TCA precipitation. (a) The plasma concentration of ^{125}I-nIgG versus time and the percentage of TCA-precipitable plasma radioactivity for nIgG. (b) The plasma concentration of ^{125}I-cIgG versus time and the percentage of TCA-precipitable plasma radioactivity for cIgG. Data are mean ± SD (n = 5).

Pharmacokinetics of cIgG, nIgG, cFab, and nFab in Rat—Plasma TCA-precipitable cIgG and nIgG concentrations are shown in Figure 2. Plasma cIgG declined with a rapid distribution followed by a relatively slow elimination with a terminal half-life of 52.9 h. However, plasma nIgG declined slowly with a terminal half-life of 81.8 h. The $AUC_{0-\infty}$ of cIgG was 116-fold lower than for nIgG. In agreement with the reduction in AUC, the Vd and the total clearance of cIgG were 74 and 114 times higher compared with those of nIgG (Table 1).

Figure 3 shows the decline of TCA-precipitable cFab and nFab in plasma. Plasma cFab decreased more rapidly than plasma nFab in the early phase of decline. The terminal half-life of cFab (27.85 ± 6.75 h) was longer than that of nFab (18.23 ± 2.46 h; p > 0.5). However, the AUC of cFab was 4-fold lower than that of nFab. Systemic clearance of cFab (162.14 ± 32.2 mL/h/kg) was 3.5-fold higher than for nFab (46.85 ± 39.33 mL/h/kg), and Vd of cFab was increased nearly 6.4-fold compared to nFab (Table 1).

The percentage of plasma TCA-precipitable radioactivity for nIgG was >96% at all times (Figure 2a), whereas that of cIgG was about 95% at the beginning, then slowly decreased to 30% at 6 h after the iv injection, then gradually increased up to a plateau of about 88% at the end of the pharmacokinetic analysis (Figure 2b). The plasma TCA-precipitable percentage of cFab and nFab also changed with time, as shown in Figures 3a and 3b. It decreased to 12 and 45% for cFab and nFab, respectively, 6 h after iv injection, and then increased again to 63 and 81%, respectively, at the end of the pharmacokinetic experiment. Autoradiography of plasma samples showed a time-dependent decrease in the intensity of the protein bands of both cationized and native antibodies, and lower molecular weight products (29−35 kDa) were observed with cIgG and nIgG.

Table 1—Pharmacokinetic Parameters of Native and Cationized IgG and Fab[a]

parameter	cIgG	nIgG	cFab	nFab	p^b cIgG–nIgG	cFab–nFab	cIgG–cFab
$t_{1/2}\lambda_d$ (h)	52.92 ± 5.2	81.84 ± 12.3	27.85 ± 6.75	18.23 ± 2.46	>0.05	>0.05	>0.05
AUC (μg/mL h)	5.3 ± 0.17	620.29 ± 92.92	4.52 ± 0.88	18.05 ± 1.64	<0.001	<0.001	>0.05
CL_t (mL/h/kg)	131.6 ± 4.4	1.15 ± 0.16	162.14 ± 32.2	46.85 ± 39.33	<0.001	<0.05	>0.05
CL_r (mL/h/kg)	2.4 ± 0.9	0.08 ± 0.05	16.58 ± 1.58	1.62 ± 0.14	<0.001	<0.001	<0.001
Vd (mL/kg)	10042 ± 864	135.9 ± 5.13	6666 ± 2196	1048 ± 211	<0.05	<0.05	>0.05

[a] In all experiments, the dose was 0.7 mg kg^{-1}; data are mean ± SD (n = 5); $t_{1/2}\lambda_d$, elimination half-life; AUC, area under the plasma concentration–time curve; CL_t, total body clearance; CL_r, renal clearance; *Vd*, volume of distribution. [b] Values are given for comparison of cIgG versus nIgG, cFab versus nFab, and cIgG versus cFab.

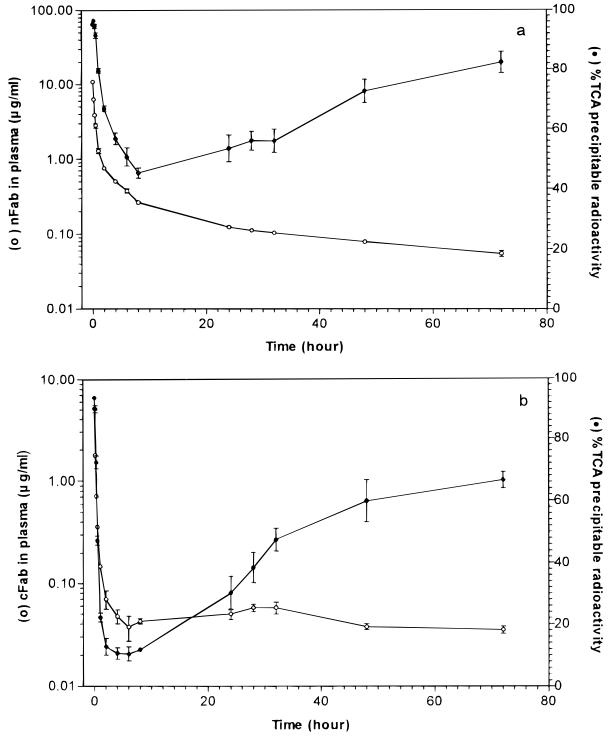

Figure 3—Plasma concentration of ^{125}I-nFab and ^{125}I-cFab. Plasma data were corrected for TCA precipitation. (a) The plasma concentration of ^{125}I-nFab versus time and the percentage of TCA-precipitable plasma radioactivity for nFab. (b) The plasma concentration of ^{125}I-cFab versus time and the percentage of TCA-precipitable plasma radioactivity for cFab. Data are mean ± SD (n = 5).

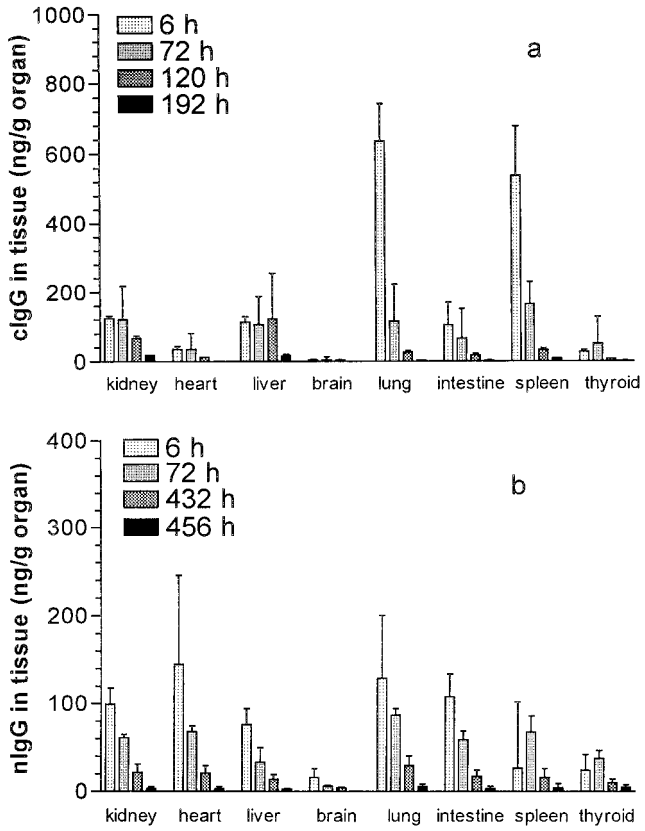

Figure 4—Biodistribution of ^{125}I-cIgG and ^{125}I-nIgG in several organs at different times after intravenous administration: (a) ^{125}I-cIgG; (b) ^{125}I-nIgG. Data are mean ± SD (n = 5).

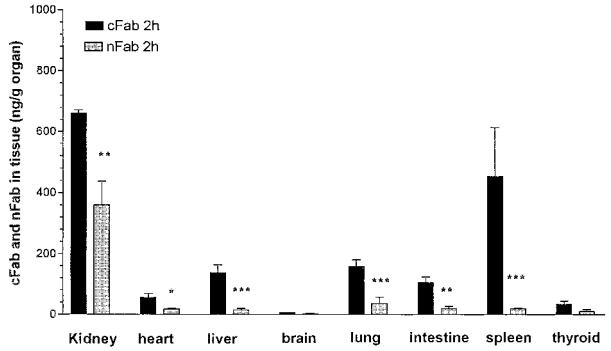

Figure 5—Biodistribution of ^{125}I-cFab and ^{125}I-nFab in several organs 2 h after iv administration. Data are mean ± SD (n = 5). Key: (*) $p < 0.05$; (**) $p < 0.01$; (***) $p < 0.001$.

Renal clearance of cIgG (2.4 ± 0.9 mL/h/kg) was only 1.8 ± 0.55% of total body clearance and 30-fold that of nIgG, which was 0.08 ± 0.05 mL/h/kg, representing 6.95 ± 4.0% of total body clearance. Renal elimination of cFab was the greatest (16.58 ± 1.58 mL/h/kg), which was 10-fold and 6.9-fold higher, respectively, than those values for nFab and cIgG (Table 1). The percentage ratios of renal clearance to the total body clearance of cFab, nFab, and cIgG were 10.2 ± 1.2, 3.46 ± 0.12, and 1.8 ± 0.8%, respectively.

The percentage of total radioactivity recovered in feces for cIgG (over 120 h), nIgG (over 456 h), cFab and nFab (both over 72 h) were 6.03 ± 0.83, 6.73 ± 0.95, 3.8 ± 0.6, and 12.08 ± 0.3%, respectively.

Organ Distribution of cIgG, nIgG, cFab and nFab in Rats—Figures 4 and 5 show the organ uptake of cIgG, nIgG, cFab, and nFab at different times following administration of antibodies. For both cationized antibodies, the organ distribution was much greater than for native antibodies. Nevertheless, this higher uptake of cationized antibodies varied between organs. Six hours after iv administration, uptake of cIgG was higher in lung and spleen (5- and 20-fold that of nIgG, respectively), whereas the uptake by kidney and liver was not so high and remained constant up to 120 h. Tissue uptake of cIgG by kidney, liver, and spleen was greatly superior to that of nIgG at all time points, whereas in lung, cIgG was

significantly increased only at 6 h ($p < 0.01$). There were no significant differences between heart, brain, intestine, or thyroid ($p > 0.05$). Tissue uptake of cIgG measured in lung, spleen, kidney, and liver at 6 h represented 0.43, 0.37, 0.08, and 0.08% of injected dose per gram of tissue, respectively. Compared with cIgG, cFab was more rapidly and extensively distributed to the organs. At 2 h, the uptake of cFab by kidney, spleen, lung, liver, intestine, and heart was greater than that of nFab, which accounted for 0.49, 0.32, 0.12, 0.10, 0.08, and 0.04% of injected dose per gram of tissue, respectively.

Discussion

One of the main problems to be solved to optimize in vivo use of antibodies is their delivery to the site of action, which can be located more deeply than the antibody distribution space. To overcome the physiological barriers limiting their diffusion, cationization based on absorptive-mediated transcytosis,[7] glycosylation,[18,19] hydrophobization,[20] and microinjection or cellular transfection[21] has been proposed for delivery of antibodies to target sites. Successful intracellular immunization of cationized anti-human immunodeficiency virus REV monoclonal antibody in human peripheral blood lymphocytes[11] and enhanced delivery of cationized immunoglobulin G across the blood-brain barrier[6] have already been described using cationized antibodies. These data encouraged us to apply the cationization concept to colchicine-specific IgG and Fab in the hope that they would be able to bind colchicine intracellularly and improve the level of detoxification already described by Chappey et al. with native colchicine-specific Fab.[9]

Following cationization, colchicine-specific IgG and Fab were resolvable into monomeric heavy and light chains with no detection of high molecular weight aggregates using SDS−PAGE. IEF confirmed that the pI of cIgG and cFab was increased to values ranging from 8.65 to 10.3 and 9.5 to 11, respectively. Moreover, there was no measurable alteration of the affinity and specificity of cIgG and cFab compared with those of nIgG and nFab. Surprisingly, we found higher cross reactivity of thiocolchicine with Fab than with either IgG. However, the cationization process cannot explain these differences because both native and cationized Fab had a similar cross reactivity. The cationized proteins were radiolabeled with ^{125}I, and the radiochemical purity and stability were similar to those of the native forms.

Radiolabeling by oxidative methods, such as the Iodogen method, could have two main drawbacks: first, the denaturation of the labeled protein and, second, the release of free iodine or smaller protein fragments labeled with iodine ^{125}I under the influence of catabolism. These drawbacks have been documented by Bauer et al.[22] who compared the pharmacokinetics of recombinant proteins with molecular weights ranging from 23 kDa to 80 kDa, which were either radiolabeled by iodine ^{125}I or quantified by specific immunoassay. This study revealed that the pharmacokinetics of proteins below about 60 kDa were different when assayed by radioactivity or immunoassay, but those proteins with a molecular weight of at least 80 kDa showed only minimal differences. A similar perturbation in the pharmacokinetics of epidermal growth factor, with a molecular weight of about 6 kDa, was attributed to molecular change in the protein configuration after radioiodination.[23] These findings had important implications for our study for at least two reasons: first, the molecular weight of Fab, about 50 kDa, is within the range of iodination side effects described by Bauer,[22] whereas IgG molecules are far above the side effects range, and, second, cationization could also have an influence on protein stability following radioiodination. For these reasons, cationized and native antibodies iodinated by the Iodogen method were measured in rat plasma by both TCA precipitation and immunoprecipitation in a satellite group of rats. More than 90% of the total radioactivity was detected by both TCA precipitation and immunoprecipitation for the four radiolabeled antibodies both before administration in the rat and in the early time in plasma (i.e., 5 min after antibody administration). These results indicate that both methods are almost equally efficient for the precipitation of radioactivity and that both radioiodinated cationized proteins can be kinetically investigated as well as radioiodinated native IgG and Fab. Nevertheless, the increased level of free ^{125}I following TCA precipitation in the distribution phase of the cIgG, cFab, and nFab kinetics suggests that the native and cationized antibodies were metabolized or deiodinated for these three compounds but not for the nIgG. The TCA-precipitable radioactivity markedly decreased and reached its lowest level at about 6−8 h after antibody administration and then increased versus time. This deiodination process could be caused by the sequestration of the native and cationized antibodies in the liver, lung, and spleen, which we have demonstrated to be the major organs of uptake (see Results section). This effect was more pronounced with the cationized than the native protein and more especially with cFab. Furthermore, urinary elimination of free iodine was maximal at 6 to 8 h after iv injection. Most of the urinary radioactivity observed with cIgG and cFab was free ^{125}I, which accounted for 98 ± 1 and 70 ± 1.7% of total radioactivity, respectively. A similar percentage of 98% of free iodine in the urinary excretion of cationized albumin has been reported by Bergmann et al.[24] However, although thyroid has a high affinity for free ^{125}I, we did not observe significant differences in iodine uptake by the thyroid for cIgG or cFab compared with nIgG and nFab. These data confirm the previous observation of Bauer[22] that high molecular weight radioiodinated proteins, such as IgG, are more stable than smaller proteins, such as Fab fragments. In addition, we also found that cationized IgG and Fab are more sensitive to deiodination than native antibodies, especially during the distribution phase. Though the TCA precipitation technique demonstrated the deiodination of the proteins, the immunoprecipitation technique showed a 2-fold decrease in the protein concentration that was similar for both cationized and native forms. This decrease could suggest that proteins were catabolized, which was not observed following SDS−PAGE autoradiography except with cIgG and nIgG for which a lower molecular weight band was observed. Nevertheless, the kinetic decline was parallel with the two techniques. These data demonstrate the analytical complexity of the analysis of radioiodinated proteins.

Another factor that could affect antibody pharmacokinetics is the immunogenicity of the native or cationized goat IgG and Fab in rats. The existence of a rat anti-goat immune response could modify antibody pharmacokinetics during the experiment. To check for such an immune response, an IRMA was used because it has been reported to be a reliable method for detection of trace amounts of protein as low as 0.013 μg/mL.[25] No rat anti-goat antibody immune response was detected following single-dose administration of either native or cationized IgG and Fab. This result can be explained by the fact that we administrated a single and relatively low dose of native and cationized antibodies. Data from the literature are variable. For example, Muckerheide et al.[26] reported that cationized bovine serum albumin (cBSA) exhibited a unique pattern of enhanced immunogenicity, and Apple et al.[27] showed that this enhanced immunogenicity was due to the increased uptake of cationized protein by antigen-present-

ing cells. In that study, the cationized antigens were administrated at high dose (100 μg/mouse) and emulsified in incomplete Freund's adjuvant. Pardridge et al.[28] reported that the repetitive administration of cationized rat albumin to rats (1 mg/kg/day for 8 weeks, sc) resulted in the development of a relatively low antibody response directed against the cationized rat albumin protein. In another study, Pardridge et al.[29] did not find any formation of antibodies directed against mouse IgG in mice treated with either native or cationized mouse IgG (7.5 mg/kg/day for 4 weeks, iv).

Taking into consideration all this methodological information, we have assessed whether the cationization process could offer significant advantages in terms of distribution and clearance between IgG or Fab. Because of the size difference between native IgG (150 kDa) and Fab (50 kDa), the volume of distribution and total clearance of native Fab are known to be higher than those of IgG because of their greater intercompartmental diffusion and glomerular filtration. Our pharmacokinetic parameters for both native molecules reflect these differences and are similar to those of previous studies,[30−32] with the exception that the terminal half-life and volume of distribution of nFab are slightly more elevated than the values most often reported. This effect could result from the persistence of a non-Fab specific radioactivity that is precipitable by TCA. In addition, our cationized antibody data clearly indicate that both cIgG and cFab were rapidly cleared from the plasma. The kinetic differences between the cationized and native forms reflect both phenomena: a marked increase in the volume of distribution, which was more pronounced for cIgG versus nIgG (ratio = 74) than for cFab versus nFab (ratio = 6.4), and in the total body clearance, which was also more pronounced for cIgG versus nIgG (ratio= 114) than for cFab versus nFab (ratio = 3.5). This higher reactivity of cIgG resulted in a volume of distribution and total body clearance that were similar to those of cFab despite its greater molecular size. The pharmacokinetic parameters of cationized and native IgG are consistent with those previously reported,[29,33,34] whereas no data were available for cFab. Based on comparison of the primary pharmacokinetic parameters, there are no marked pharmacokinetic differences between cIgG and cFab, except for the higher renal clearance of cFab (Table 1).

Even if increased transport to the extracellular fluid is in part responsible for the higher distribution and clearance of cIgG and cFab, it cannot be the only mechanism. The extracellular fluid volume in rat is approximately 240 mL/kg,[30] so the *Vd* values of cIgG and cFab are, respectively, 41- and 28-fold greater than the extracellular fluid volume, suggesting extensive tissue interactions and possible endocytosis. Thus, the rapid removal of cIgG and cFab from the plasma could be attributed to several mechanisms, such as increased escape to the extracellular space, binding to cells and basement membrane, intracellular uptake, and degradation or elimination in urine and feces. All these mechanisms are compatible with the observed increase in volume of distribution and total body clearance of cationized IgG and Fab.

The binding of cationized proteins to cells and basement membrane has already been demonstrated.[18,29] However, we have also observed enhanced intracellular uptake of colchicine-specific cIgG and cFab compared with nIgG and nFab by isolated parenchymal and nonparenchymal cells of the rat liver[35]and also in HL60 cells and human peripheral lymphocytes (results not given). Our data confirm the greater organ interaction of cIgG and cFab, although the distribution pattern was not exactly the same for cIgG and cFab. cIgG was predominantly taken up by lung and spleen, whereas kidney and spleen were more interactive with cFab; and, with both cationized compounds, liver uptake was also enhanced, whereas no or only moderate enhancement was observed in brain, thyroid, and intestine. This organ distribution profile is in agreement with previous studies,[7,29] except that we found a greater cIgG uptake in lung and spleen. These observations suggest that the transcapillary or transcellular passage of cationic proteins is an organ- and protein-specific process. The morphology of the vascular endothelium, which varies from organ to organ and from one location to another within the vasculature of the same organ,[33] could explain the differences in uptake of cationized proteins by different organs. Uptake of both cFab and cIgG was higher than that of the native forms in liver, which is to be expected because liver is an important interactive organ for cationic macromolecules.[36] Receptors for positively charged proteins have been identified on Kupffer cells as well as on endothelial cells.[24,37] In contrast to other organs in which the capillaries present a substantial barrier between vascular and interstitial spaces, the discontinuous endothelial capillaries of the liver favor the electrostatic interaction of cationic proteins with the negative charges of the liver cell surfaces.[36] A substance with a net cationic charge can reach a 4-fold higher intracellular concentration due to the inside negative membrane potential of the hepatocyte (−30 to 40 mV).[38] This effect might be the main explanation for the higher liver uptake of cIgG and cFab than of nIgG and nFab. In contrast, the poor enhancement of brain uptake of both cationized antibodies could be explained by the special anatomical features of the endothelial cells in brain capillaries. The tight junctions linking the endothelial cells of the cerebral vessels form a continuous endothelium that does not seem as favorable as fenestrated or discontinuous endothelium for the uptake of cationized antibodies.

In addition to these distribution properties, several clearance mechanisms can be observed with both cationized antibodies. First, renal clearance was enhanced for cationized proteins compared with native forms. The glomerular capillary wall functions as a barrier based on discrimination of molecular size and electric charge.[39] Normally, this wall restricts almost completely the passage of compounds with molecular weight >50 kDa[40] such as IgG. In contrast, the 50 kDa Fab is filtered and partially reabsorbed at the level of the proximal tubule,[41] and then undergoes extensive catabolism into peptides that recirculate in the blood. Little information is available concerning the influence of electric charge. Filtration of negatively charged dextran sulfate (64 kDa) is greatly restricted, whereas the positively charged diethylaminoethyldextran (DEAED, molecular weight 64 kDa) shows enhanced ability to cross the glomerular wall compared with neutral dextrans of similar size.[40] We found increased kidney distribution and renal clearance for both cIgG and cFab, in which renal clearance of cFab accounted for 10% of total body clearance.

Clearance could also be dependent on the degradation process affecting the protein itself and on iodine release. These last effects have been discussed by comparing the TCA and immunoprecipitation data.

In conclusion, this study demonstrates that cationization of both colchicine-specific polyclonal IgG and Fab fragments increased their organ distribution in vivo and markedly altered their plasma pharmacokinetics compared with the native compounds. These pharmacokinetic properties give new insights into the increased detoxification capability of these cationized antibodies. Colchicine has been reported to be ubiquitously distributed, with high accumulation in spleen, kidney, lung, liver, and heart.[42] Because cIgG and cFab also distribute within these tissues, cationized specific

IgG and Fab could be more effective at reversing colchicine toxicity because of their more extensive distribution to the intracellular colchicine action site within the tissues.

References and Notes

1. Waldmann, T. A. Monoclonal Antibodies in Diagnosis and Therapy, *Science* **1991**, *252*, 1657−1662.
2. Scherrmann, J. M.; Urtizberea, M.; Pierson; P.; Terrien, N. The Effect of Colchicine-Specific Active Immunization on Colchicine Toxicity and Disposition in the Rabbit. *Toxicology* **1989**, *56*, 213−222.
3. Brady, L. W.; Markoe, A. M.; Woo, D. V. Iodine-125-labeled Anti-epidermal Growth Factor Receptor-425 in the Treatment of Glioblastoma Multiforme. A Pilot Study. *Radiat. Ther. Oncol.* **1990**, *24*, 151−160.
4. Jain, R. K. Tumor Physiology and Antibody Delivery. *Radiat. Ther. Oncol.* **1990**, *24*, 32−46.
5. Pardridge, W. M. Absorptive-Mediated Transcytosis of Peptides Through the Blood-Brain Barrier. In *Peptide Drug Delivery to the Brain*; Raven: New York, 1991; pp 189−218.
6. Triguero, D.; Buciak, J. L.; Pardridge, W. M. Blood-Brain Barrier Transport of Cationized Immunoglobulin G. Enhanced Delivery Compared to Native Protein. *Proc. Natl. Acad. Sci., U.S.A.* **1989**, *86*, 4761−4765.
7. Triguero, D.; Buciak, J. L.; Pardridge, W. M. Cationization of Immunoglobulin G Results in Enhanced Organ Uptake of the Protein after Intravenous Administration in Rats and Primate. *J. Pharmacol. Exp. Ther.* **1991**, *225*, 186−192.
8. Pardridge, W. M. Transport of Small Molecules through the Blood-brain Barrier: Biology and Methology. *Adv. Drug Delivery Rev.* **1995**, *15*, 5−36.
9. Chappey, O. L.; Niel, E.; Debray, M.; Wautier, J. L.; Scherrmann, J. M. Efflux of Intracellular Colchicine in Lymphocytes with Colchicine-specific Fab Fragments. *J. Pharmacol. Exp. Ther.* **1995**, *274*, 1072−1076.
10. Sabouraud, A. E.; Urtizberea, M.; Benmoussa, K. Fab-bound Colchicine Appears to Adopt Fab Fragment Disposition in Rats. *J. Pharm. Pharmacol.* **1992**, *44*, 1015−1019.
11. Pardridge, W. M.; Bickel, U.; Buciak, J.; Yang, J.; Diagne, A.; Aepinus, C. Cationization of a Monoclonal Antibody to the Human Immunodeficiency Virus REV Protein Enhances Cellular Uptake but Does Not Impair Antigen Binding of the Antibody. *Immunol. Lett.* **1994**, *42*, 191−195.
12. Laemmli, U. K. Cleavage of Structural Proteins During the Assembly of the Head of Bacteriophage T_4 *Nature (London)* **1970**, *277*, 680−685.
13. Müller, R. Calculation of Average Antibody Affinity in Anti-Hapten Sera from Data Obtained by Competitive Radioimmunoassay. *J. Immunol. Meth.* **1980**, *34*, 345−352.
14. Fraker, P. J.; Speck, J. C. Protein and Cell Membrane Iodinations with a Sparingly Soluble Chloramide 1,3,4,6-Tetrachloro-3a, 6a-Diphenylglycoluril. *Biochem. Biophys. Res. Commun.* **1978**, *80*, 849−857.
15. Pardridge, W. M. Carrier-Mediated Transport of Thyroid Hormones Through the Blood-Brain Barrier. Primary Role of Albumin-Bound Hormone. *Endocrinology* **1979**, *105*, 605−612.
16. Ebling, W. F.; Wada, D. R.; Stanski, D. R. From Piecewise to Full Physiologic Pharmacokinetic Modeling: Applied to Thiopental Disposition in the Rat. *J. Pharmacokinet. Biopharm.* **1994**, *22* (*4*), 259−292.
17. Gibaldi, M.; Perrier, D. In *Pharmacokinetics*; Gibaldi, M.; Perrier, D.; Eds.; Dekker: New York, 1982.
18. Smith, K. R.; Borchardt, R. T. Permeability and Mechanism of Albumin, Cationized albumin, and Glycosylated Albumin Transcellular Transport Across Monolayers of Cultured Bovine Brain Capillary Endothelial cells. *Pharm. Res.* **1989**, *6*, 466−473.
19. Williams, S. K.; Devenny, J. J.; Bitensky, M. W. Micropinocytic Ingestion of Glycosylated Albumin by Isolated Microvessels: Possible Role in Pathogenesis of Diabetic Microangiopathy. *Proc. Natl. Aacad. Sci., U.S.A.* **1981**, *78*, 2393−2397.
20. Chekhonin, V. P.; Ryabukhin, I. A.; Zhirkov, Y. A. Transport of Hydrophobized Fragments of Antibodies through the Blood-Brain Barrier. *Neuroreport* **1995**, 129−132.
21. Mulcahy, L. S.; Smith, M. R.; Stacey, D. W. Requirement for Ras Proto-Oncogene Function during Serum-Stimulated Growth of NIH 3t3 Cells. *Nature* **1985**, *313*, 241−243.
22. Bauer, R. J.; Leigh, S. D.; Birr, C. A.; Bernhard, S. L. Alteration of the Pharmacokinetics of Small Proteins by Iodination. *Biopharm. Drug. Dispos.* **1996**, *17*, 761−774.
23. Kuo, B.; Nordblom, G. D.; Wright, D. S. Pertubration of Epidermal Growth Factor Clearance after Radioiodination and Its Implication. *J. Pharm. Sci.* **1997,** *86* (*13*), 290−296.
24. Bergmann, P.; Kacenelenbogen, R.; Vizet, A. Plasma Clearance, Tissue Distribution and Catabolism of Cationized Albumins with Increasing Isoelectric Points in the Rat. C*lin. Sci.* **1984**, *67*, 35−43.
25. Pepin, S.; Lutsch, C.; Grandgeorge, M.; Scherrmann, J. M. Snake F(ab′)2 Antivenom from Hyperimmunized Horse: Pharmacokinetics Following Intravenous and Intramuscular Administrations in Rabbits. *Pharm. Res.* **1995**, *12* (*10*), 1−4.
26. Muckerheide, A.; Domen, P. L.; Michael J. G. Cationisation of Protein Antigens. II. Alteration of regulatory properties. *J. Immunol.* **1987**, *138* (*9*), 2800−2804.
27. Apple, R. J.; Domen, P. L.; Muckerheide, A.; Michael J. G. Cationisation of Protein Antigens. III. Increased Antigen Uptake by Antigen-Presenting Cells. *J. Immunol.* **1988**, *140* (*10*), 3290−3295.
28. Pardridge, W. M.; Triguero, D.; Buciak, J. L.; Yang, J. Evaluation of Cationized Rat Albumin as a Potential Blood-Brain Barrier Drug Transport Vector. *J. Pharmacol. Exp. Ther.* **1990**, *255* (*2*), 893−899.
29. Pardridge, W. M.; Kang, Y.; Yang, J.; Buciak, J. L. Enhanced Cellular Uptake and in vivo Biodistribution of a Monoclonal Antibody Following Cationization. *J. Pharm. Sci.* **1995**, *84*, 943−948.
30. Pentel, P. K.; Keyler, D. E.; Gilbertson, D. G.; Ruth, G.; Pond, S. M. Pharmacokinetics and Toxicity of High Doses of Antibody Fab Fragments in Rats. *Drug Metab. Dispos.* **1988**, *16*, 141−145.
31. McClurkan, M. B.; Valentine, J. L.; Arnold, L., Owens; S. M. Disposition of Monoclonal Anti-phencyclidine Fab Fragments of Immunoglobulin G in Rats. *J. Pharmacol. Exp. Ther.* **1993**, *266***,** 1439−1445**.**
32. Bazin-Redureau, M. I.; Renard, C. B.; Scherrmann, J. M. G. Pharmacokinetics of Heterologous and Homologous Immunoglobulin G, F(ab′)2 and Fab after Intravenous Administration in the Rat. *J. Pharm. Pharmacol.* **1997**, *49*, 277−281.
33. Pardridge, W. M.; Buciak, J. L.; Friden, P. M. Selective Transport of an Anti-Transferrin Receptor Antibody through the Blood-Brain Barrier. *J. Pharmacol. Exp. Ther.* **1991**, *252*, 1247−1254.
34. Pardridge, W. M.; Kang, Y.; Diagne, A.; Zack, J. A. Cationized Hyperimmune Immunoglobulins: Pharmacokinetics, Toxicity Evalution and Treatment of Human Immunodeficiency Virus-Infected Human-Peripheral Blood Lymphocytes-Severe Combined Immune Deficiency Mice. *J. Pharmacol. Exp. Ther.* **1996**, *276*, 246−252.
35. Hong, G.; Bazin-Redureau, M. I.; Scherrmann, J. M. G. Hepatic Disposition and Toxicity of Cationized Goat Immunoglobulin G and Fab Fragments in Isolated Perfused Rat Liver. *Drug Metab. Dispos.*, in press.
36. Takakura, Y.; Fujita, T.; Hashida, M.; Sezaki, H. Disposition Characteristics of Macromolecules in Tumor-Bearing Mice. *Pharm. Res.* **1990**, *7*, 339−346.
37. Praaning-van Dalen, D. P.; Brouwer, A.; Knook, D. L. Clearance Capacity of Rat Liver Kupffer, Endothelial, and Parenchymal Cells. *Gastroenterology* **1981,** *81*(*6*), 1036−1044.
38. Meiger, D. K. F.; Mol, W. E. M.; Muller, M.; Kurz, G. Carrier-mediated Transport in the Hepatic Distribution and Elimination of Drugs, with Special Reference to the Category of Organic Cations. *J. Pharmacokinet.* **1990**, *18*, 35−70.
39. Taylor, A. E.; Granger, N. D. Exchange of Macromolecules Across the Microcirculation; In *Handbook of Physiology: the Cardiovascular System*, Section 2, Volume IV; Renkin, E. M.; Michel, C. C., Eds.; American Physiology Society: Bethesda, MD, 1984; pp 467−520.
40. Brenner, B. M.; Hostetter, T H.; Humes, H. D. Glomerular Permeselectivity: Barrier Function Based on Discrimination of Molecular Size and Charge. *Am. J. Physiol.* **1978**, *234*, 7455−7460.
41. Arend, W. P.; Silverblatt, F. J. Serum Disappearance and Catabolism of Homologous Immunoglobulin Fragments in Rats. *Clin. Exp. Immunol.* **1975**, *22* (*3*), 502−513.
42. Rochdi, M.; Sabouraud, A.; Baud, F. J.; Bismuth, C.; M., S. J. Toxicokinetics of Colchicine in Humans: Analysis of Tissue, Plasma and Urine Data in Ten Cases. *Hum. Exp. Toxicol.* **1992**, *11*, 510−516.

Acknowledgments

This work was supported by a grant from INSERM/Ministère de la Défense.

JS970335N