HEPATIC DISPOSITION AND TOXICITY OF CATIONIZED GOAT IMMUNOGLOBULIN G AND FAB FRAGMENTS IN ISOLATED PERFUSED RAT LIVER

G. HONG, M. BAZIN-REDUREAU, P. GIRES, AND J. M. SCHERRMANN

INSERM U26 Département de Pharmacocinétique de la Faculté de Pharmacie (G.H., M.B.-R., J.M.S.) and Département de Biodynamique et de Métabolisme et Pharmacocinétique, Rhône Poulenc-Rorer (P.G.)

(Received November 4, 1997; accepted March 16, 1998)

This paper is available online at http://www.dmd.org

ABSTRACT:

Colchicine-specific goat IgG and Fab fragments were cationized by covalent coupling of hexamethylenediamine. The immunoreactivity of antibodies was not changed following cationization. The interaction of 125I-radiolabeled native (nIgG and nFab) and cationized immunoglobulin G (cIgG and Fab fragments (cFab) with liver was investigated using isolated perfused rat liver (IPRL) and isolated rat hepatic parenchymal cells (PCs) and nonparenchymal cells (NPCs) in suspension. 125I-cIgG or 125I-cFab were more rapidly cleared from the perfusate than the corresponding native proteins. Both cIgG and cFab declined biexponentially over time in the perfusate. In contrast, the native IgG and Fab decreased monoeXponentially. The half-lives of the initial and terminal phases were 5.2 ± 1.6 min and 355.1 ± 17.2 min for cIgG and 14.7 ± 3.4 min and 552.4 ± 23.7 min for cFab. The terminal half-lives of nIgG (467.4 ± 11.6 min) and nFab (880.1 ± 39.6 min) were longer than those of cationized molecules. The biliary protein extraction ratio of cationized IgG and Fab was greater than that of native IgG and Fab: 0.13% (cIgG), 0.02% (nIgG), 0.23% (cFab), and 0.17% (nFab). The uptake of cIgG and cFab by both PCs and NPCs was dose-dependent and was about 6-fold and 8-fold higher than that of their native counterparts, respectively. Throughout the experiment, liver viability was determined, and no toxicity was observed according to physiological analysis (bile flow rate, portal vein pressure, and pH) and biochemical analysis (glucose and hepatic enzymes: alanine transaminase, aspartate transaminase, lactate dehydrogenase) in perfusate.

Antibodies and their Fab fragments directed toward circulating or noncirculating antigens are widely used in immunodiagnosis and immunotherapy (Bickel, 1995; Takakura, 1996). However, antibodies like other large plasma proteins are poorly transported across cell membranes or capillary barriers in vivo and are generally confined to the plasma compartment in the case of whole immunoglobulin (Dewey, 1959). The size-limited diffusion of antibodies is the main factor limiting their interaction with more deeply distributed antigens. One possible strategy for the intracellular delivery of macromolecules is their cationization (Triguerdo et al., 1989), in which the anionic side chain carboxyl groups of the protein are conjugated with polycationic aminoethylamide groups resulting in an increase in the protein isoelectric point (pl). 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 (Kumagai et al., 1987; Partridge et al., 1990; Shimura et al., 1991; Terasaki et al., 1989, 1991, 1992). Thus, antibody could enter cells and bind to intracellular antigens that would not normally be accessible to antibodies.

Several reports have examined the pharmacokinetics of cationized immunoglobulin IgG in vivo (Partridge et al., 1994, 1995), but few data are available concerning the pharmacokinetics of cationized Fab fragments. The liver has been described as one of the most active organs for antibody catabolism (Fukumoto and Brandon, 1982; Jones et al., 1990). For instance, Covell et al. (1986) showed that the gut accounted for 72.8% of total catabolism of murine IgG in mice, followed by the liver (20.5%) and then the spleen (3.6%). Partridge et al. (1995) studied the pharmacokinetics of a cationized murine monoclonal antibody in vivo, but little is known about the hepatic disposition of cationized antibody. In a previous study, we used the isolated perfused rat liver model (IPRL), which allows demonstration of very low hepatic extraction ratio (<0.01) for the native form of IgGs and Fab fragments (Bazin-Redureau et al., 1995). In the present study, the hepatic uptake of cationized IgG and Fab was investigated using the same model, and the hepatic toxicity of these modified proteins was simultaneously monitored by measurement of physiological and biological hepatic markers. Finally, in vitro interaction with isolated rat PCs and NPCs was assessed to characterize the binding properties of the cationized antibodies to both liver cell types.

Materials and Methods

Goat IgG and Fab fragments of colchicine-specific polyclonal antibody were prepared as previously described (Sabouraud et al., 1992). IgG and Fab fragments were purified by gel chromatography to 97 and 92%, respectively. Colchicine (Mₙ = 399) and thiocolchicine (Mₙ = 564) were obtained from Roussel Uclaf (Paris), and [³H]colchicine (Ring C, [³H]methoxy, 66 Ci/mmol) was from New England Nuclear (Paris). Hexamethylenediamine, N-ethyl-N'...
(3-dimethylamino)propyl)carbodiimide, glycine, and others reagents for protein cationization were obtained from Sigma (St. Quentin Fallavier, France).

Cationization of Colchicine-Specific IgG and Fab. Native colchicine-specific IgG and Fab were cationized according to the method of Pardridge et al. (1994), with small modifications. Ten milligrams of native IgG and Fab were slowly added to 4 ml of 2 M hexamethylenediamine (pH 6.2). To this mixture, 50 mg of fresh N-ethyl-N’-(3-dimethylamino)propyl)carbodiimide was added, and the pH was adjusted to 6.20. The mixture was stirred for 3 hr at room temperature, and the reaction was quenched by addition of 2 M glycine followed by incubation for 60 min at room temperature. The mixture was then dialyzed overnight at 4°C against 0.01 M Na2HPO4, 0.15 M NaCl (pH 7.4). The precipitate was removed by centrifugation (1000 g, 10 min), and the solution was concentrated with polyethylene glycol (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 was determined by isoelectric focusing (Triguero et al., 1989). Thirty micrograms of protein samples were dialyzed with 0.05% glycine overnight at 4°C to eliminate excess ions and analyzed by isoelectric focusing according to the manufacturer’s instructions (Sigma). After isoelectric focusing, the gel was stained with Coomassie Blue. SDS-PAGE was also performed to determine the molecular weight of the cationized and native antibodies.

The affinity constant was determined by competitive radioimmunoassay according to the method of Müller (1980). Specificity of the native and cationized antibodies was measured by the cross-reactivity of a colchicine analogue, thiocolchicine, using radioimmunoassay as previously described by Scherrmann et al. (1989).

Radiolabeling of IgG and Fab. Antibodies were labeled using the Iodogen method (Fraker and Speck, 1978). One hundred micrograms of protein were incubated with 0.5 mCi of 125I-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). Trichloroacetic acid precipitability fraction was greater than 95% for the iodinated preparations. The specific activity was in the range of 2–3 μCi/μg for native IgG, 1–2 μCi/μg for cIgG, 1–2 μCi/μg for native Fab, and 0.6–1.5 μCi/μg for cFab. After radiolabeling, cIgG, nIgG, cFab, and nFab were analyzed every day for 1 week by SDS-PAGE autoradiography to determine their stability.

Cationized and Native Antibody Uptake by the Isolated Perfused Rat Liver. Male Sprague-Dawley rats (Iffa Credo, Lyon, France) weighing 250–300 g were used. They had free access to standard laboratory chow and tap water. Rats were anesthetized with ether, and livers were isolated and perfused with recirculation of the perfusion medium according to Brauer et al. (1951) with modifications. Rats were given heparin (100 IU) via the penile vein. The liver was exposed and the bile duct cannulated with PE-10 tubing (Biotrol, Paris). The portal vein was cannulated with PE-200 tubing, the liver was freed from attachment to the diaphragm, and the vena cava was severed. The liver was transferred to a thermostatically controlled Plexiglas chamber (37°C) where a cannula attached to the perfusion system was secured in the portal vein. The outflow of the perfusate from the vena cava was collected in a reservoir. The perfusate (120 ml) consisted of 40 ml of rat donor blood, 80 ml of 4.5% bovine serum albumin in Krebs-Ringer bicarbonate solution (pH 7.4) to give a hematocrit of 12%. Perfusate oxygenated with O2 :CO2 (95:5%) was recirculated at a mean flow rate of 50 ml/min with a Masterflex pump (Bioblock, Paris) over a 3-hr period. A solution of 0.5 M NaHCO3, 3 mM sodium taurocholate, and 5 g/liter glucose was continuously infused into the reservoir at a flow rate of 0.015 ml/min to maintain the perfusate at pH 7.4. Temperature and pH of the perfusate, portal vein pressure, and bile flow were continuously monitored. Biochemical assays of liver viability were performed in the erythrocyte-free perfusate with a centrifugal analyzer and included

FIG. 1. Time course of 125I-cIgG in perfusate.
determination of glucose (Glucostat kit, Boehringer), lactate dehydrogenase (LDH) (Enzyme LDH/HBDH kit, BioMérieux), aspartate transaminases (AST) (Chiron Diagnostics, Cergy Pontoise, France), and alanine transaminases (ALT) (Chiron Diagnostics).

The liver was allowed to equilibrate for 1 hr, during which viability assays were performed before adding 40 μg, 15 μCi of 125I-cIgG, -nIgG, -cFab, and -nFab (N = 4) to the perfusate.

Bile was collected in preweighed vials at 0–10-, 11–20-, 21–30-, 31–40-, 41–50-, 51–75-, 76–105-, 106–135-, and 136–180-min intervals after protein injection. Perfusate samples were collected at 1, 3, 5, 7, 10, 15, 20, 25, 30, 40, 50, 60, 75, 90, 105, 120, 135, 150, and 180 min after protein injection and were centrifuged immediately to separate the RBC from perfusate. Bile, erythrocyte-free perfusate, and erythrocyte pellet samples were counted for radioactivity in a gamma counter. After counting, an aliquot was assayed for trichloroacetic acid-precipitable radioactivity and analyzed by SDS-PAGE followed by autoradiography.

**Cationized and Native Antibody Uptake by Isolated Rat Cells.** Rat liver cells were isolated according to the collagenase perfusion method of Berry and Friend (1969), with modifications. Rats were anesthetized by an intraperitoneal injection of pentobarbital sodium (Clin-Midy, France). The liver was first perfused via the portal vein with Ca²⁺-free HEPES solution (137 mmol/liter NaCl, 2.7 mmol/liter KCl, 0.28 mmol/liter Na₂HPO₄ H₂O, and 10 mmol/liter HEPES) for 7 min at a flow rate of 28 ml/min (pH 7.4; temperature 37°C) and then with HEPES solution containing 0.05% collagenase (Boehringer Mannheim, Germany) and 5 mM CaCl₂ for <15 min at a flow rate of 15 ml/min. After stopping the perfusion, the liver was excised, and the cells were dispersed by gentle stirring in an L15 medium of 0.2% BSA. The cell suspension was filtered through 40-μm nylon mesh and then centrifuged at 50g for 2 min. The pelleted cells (mainly hepatocytes or PCs) were resuspended and washed three times in an L15 medium of 0.2% BSA. NPCs (principally endothelial cells and Kupffer cells) remaining in the initial 50g supernatant were sedimented at 400g for 4 min. The cell pellet was resuspended in Hank’s balanced salt solution and centrifuged at 50g for 2 min to remove the remaining parenchymal cells. The supernatant was then sedimented at 400g for 4 min. The cell pellet was resuspended in 5 ml of Gey’s balanced saline solution and mixed with 7 ml of 29% nycodenz; 29% nycodenz was prepared in NaCl-free Gey’s balanced saline solution. Red blood cells and cell debris were sedimented by centrifugation at 1400g for 15 min. The cell layer on top of the gradient was considered to be the NPC fraction (Van Bossuyt et al., 1988). NPCs were washed three times in Hank’s balanced salt solution.

Cells were incubated for 30 min in RPMI containing 20 mM HEPES and 1% BSA (pH 7.4) at 37°C in a 95% O₂, 5% CO₂ atmosphere to permit recovery of cells.

Cell viability was assessed both by the exclusion of 0.5% trypan blue and by the release of LDH into the medium and was estimated to be higher than 90%. Uptake experiments (N = 4 for each protein) were conducted in plastic tubes presoaked with 0.2% BSA. Cells (5 × 10⁶ cells/ml for PCs and 2 × 10⁶ cells/ml for NPCs) were incubated at 37°C in 10 mM HEPES-buffered Hank’s buffer (pH 7.4) in a total volume of 0.2 ml for 2 hr.

Assays were started by addition of 125I-labeled and nonlabeled antibody (cIgG, nIgG, cFab, nFab at a concentration of 0.01, 0.1, 1, 10, and 20 μg/ml, respectively). To determine cell association radioactivity, 170 μl were removed and placed in 500-μl microtubes containing 190 or 100 μl of a mixture of dinonyl phthalate and dibutyl phthalate; the ratio of dinonyl phthalate to dibutyl phthalate was one-half (v/v) for hepatocytes and one-third for NPCs.

Cells were centrifuged for 3 min at 10,000g at 4°C. Only the viable cells passed through the oil. Cell pellets formed in the bottom of the tube, and the supernatant was carefully removed. After washing three times with Hank’s balanced salt solution, the bottom of the tube containing cell pellets was cut off and placed in a plastic tube for radioactivity determination in a gamma counter.

---

**FIG. 2.** Time course of 125I-nIgG in perfusate.

Each error bar represents the mean ± SD (N = 4). (a) The computer-fitted time profile of the percentage of nIgG remaining in the perfusate. (b) The cumulative biliary excretion of nIgG and free iodine.
Table 1: Pharmacokinetic parameters of cIgG, nIgG, cFab, and nFab in IPRL.

Data are mean ± SE (N = 4). \( t_{1/2} \), half-life; \( V_p \), volume of distribution; \( CL_h \), apparent hepatic uptake clearance; \( \text{AUC}_5 \), percent of the initial phase AUC to the total AUC; \( E \), hepatic extraction ratio. The statistical differences between cIgG and nIgG, cFab and nFab, and cIgG and cFab are shown as \( p \) values.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>cIgG</th>
<th>nIgG</th>
<th>cFab</th>
<th>nFab</th>
<th>p cIgG-nIgG</th>
<th>p cFab-nFab</th>
<th>p cIgG-cFab</th>
</tr>
</thead>
<tbody>
<tr>
<td>Perfusion</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>( t_{1/2a} ) (min)</td>
<td>5.216 ± 1.629</td>
<td>—</td>
<td>14.72 ± 3.38</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>0.0445</td>
</tr>
<tr>
<td>( t_{1/2b} ) (min)</td>
<td>355.07 ± 17.25</td>
<td>467.44 ± 11.63</td>
<td>552.42 ± 23.70</td>
<td>880.15 ± 39.56</td>
<td>&lt;0.01</td>
<td>&lt;0.01</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>( V_p ) (ml)</td>
<td>198.66 ± 18.92</td>
<td>130.7 ± 10.5</td>
<td>204 ± 12.37</td>
<td>131.9 ± 0.6</td>
<td>&lt;0.05</td>
<td>&lt;0.05</td>
<td>&gt;0.05</td>
</tr>
<tr>
<td>( CL_h ) (ml/min)</td>
<td>0.425 ± 0.03</td>
<td>0.2 ± 0.00</td>
<td>0.275 ± 0.0068</td>
<td>0.1 ± 0.001</td>
<td>&lt;0.01</td>
<td>&lt;0.0001</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>AUC (_0-\infty) (min( \times )mg/ml)</td>
<td>96.91 ± 11.70</td>
<td>196.51 ± 33.48</td>
<td>84.47 ± 7.80</td>
<td>219.41 ± 39.12</td>
<td>&lt;0.05</td>
<td>&lt;0.01</td>
<td>&gt;0.05</td>
</tr>
<tr>
<td>( E )</td>
<td>0.0085 ± 0.0007</td>
<td>0.004 ± 0.0001</td>
<td>0.0055 ± 0.00013</td>
<td>0.002 ± 0.0001</td>
<td>&lt;0.01</td>
<td>&lt;0.0001</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Bile</td>
<td>% of dose</td>
<td>0.13 ± 0.001</td>
<td>0.018 ± 0.002</td>
<td>0.223 ± 0.04</td>
<td>0.17 ± 0.009</td>
<td>&lt;0.0001</td>
<td>&lt;0.05</td>
</tr>
</tbody>
</table>

Fig. 3. Time course of \( 125\)I-cFab in perfusate.

Each error bar represents the mean ± SE (N = 4). (a) The computer-fitted time profile of the percentage of cFab remaining in the perfusate. (b) The cumulative biliary excretion of cFab and free iodine.

In Vitro Interaction of Cationized Antibody with Bovine Serum Albumin. SDS-PAGE was used to determine whether cationized antibodies interacted with bovine serum albumin. BSA and antibodies were mixed (30:1) and incubated at 37°C for 2 hr, which is compatible with detection of BSA and cationized antibodies. BSA, cIgG, nIgG, cFab, and nFab were used as controls. SDS-PAGE was performed under no-reduction condition according to Laemmli (1970).

Kinetic and Statistical Analyses. The TCA-precipitable cationized and native IgG and Fab concentration in the erythrocyte-free perfusate and erythrocyte pellet were fitted to a two-compartment or one-compartment open model using nonlinear regression by extended least squares analysis (Siphar, Simed, Créteil, France). To assess the "goodness of fit," residual analysis (an examination of the standard deviation) was performed. In addition to the likelihood test, Akaike criteria were tested to select the most appropriate model (Yamokura et al., 1978). Pharmacokinetic parameters were calculated by fitting the data to a biexponential (\( C = Ae^{-\lambda_1 t} + Be^{-\lambda_2 t} \)) or a monoexponential equation (\( C = Ae^{-\lambda t} \)), where \( A \) and \( B \) are the extrapolated concentrations at \( t = 0 \), and \( \lambda_1 \) or \( \lambda_2 \) are the protein elimination rate constants. The corresponding half-lives were calculated as \( 0.693/\lambda_1 \) or \( \lambda_2 \). The area under the perfusate concentration-time curve from zero to infinity (\( \text{AUC}_0-\infty \)) was calculated as \( \text{AUC}_0-\infty = A/\lambda_1 + B/\lambda_2 \) or as \( A/\lambda_1 \) (monoexponential decay). Distribution volume (\( V_p \)) and hepatic clearance (\( CL_h \)) were determined as follows: \( V_p = \text{dose} / \text{AUC}_0-\infty \) and \( CL_h = \text{dose} / \text{AUC}_h \). The hepatic extraction ratio (\( E \)) was calculated as \( E = CL_h / Q \), where \( Q \) is the perfusate flow rate.

The amount of \( 125\)I excreted in each bile sample was calculated by multiplying the sample volume by the concentration. The cumulative amount of \( 125\)I at time \( t \) (\( B \)) and at time \( t_e \) (\( B_e \)) was calculated from this value. Results are
Results

Characteristics of Antibodies after Cationization. After cationization, cIgG migrated with a pI of 8.7–10.3 in contrast to 5.9–9.0 for nIgG; cFab migrated with a pI of 9.5–11.0 in contrast to 8.6–9.3 for nFab. However, cationization did not significantly alter the affinity and specificity of IgG and Fab. The affinity constants for colchicine of cIgG and nIgG were, respectively, 1.2 ± 0.13 × 10^9 and 1.58 ± 0.16 × 10^9 M^-1 (p > 0.05), and 1.08 ± 0.21 × 10^9 and 0.64 ± 0.18 × 10^9 M^-1 (p > 0.05) for cFab and nFab. The cross-reactivity to thiocolchicine did not differ significantly between cationized and native forms. The values for cIgG and nIgG were 8.6 ± 1.13% and 8.9 ± 0.58% (p > 0.05), respectively, and for cFab and nFab 25 ± 5.9% and 37 ± 8.67% (p > 0.05), respectively. Furthermore, migration of cationized and native antibodies in SDS-PAGE did not show any measurable alteration for up to 1 week after iodination.

In Vitro Interaction of Cationized Antibody with Bovine Serum Albumin. The inhibitory role of binding of both cationized antibodies to serum albumin in the perfusate was assessed by SDS-PAGE because the isoelectric point of albumin is close to 4 at pH 7.4, allowing possible interaction between cationized proteins and albumin. The bands that appeared in the BSA-cIgG and BSA-cFab mixtures were the same as with nIgG and nFab. These bands corresponded to BSA, IgG, and Fab.

These results might indicate that 1) there was no complex formation between BSA-cIgG and BSA-cFab or 2) the amount of complex was too small to be detected by SDS-PAGE, even under the no-reduction condition. If the latter is the case, the low affinity between BSA-cIgG or BSA-cFab should favor the dissociation of the cationized proteins from BSA to the surface of the hepatocyte, which has a high inside negative membrane potential (~30 to 40 mV) (Meijer et al., 1990). In view of these in vitro data, binding of cationized proteins to the perfusate proteins was considered to be nil in the liver uptake experiments.

Uptake of Cationized and Native Colchicine-Specific Antibodies by IPRL. Erythrocyte-free perfusate cIgG concentrations declined biexponentially with initial and terminal half-lives of 5.2 ± 1.6 min and 355 ± 17.2 min, respectively (fig. 1), whereas the disappearance of nIgG from the perfusate medium was characterized by a monoexponential decay with a half-life of 467 ± 11 min (fig. 2). Kinetic parameters are presented in table 1. The volume of distribution was significantly different between cIgG (198.7 ± 18.9 ml) and nIgG (130.7 ± 10.5 ml) (p < 0.05). The hepatic clearance of cIgG and nIgG was 0.43 ± 0.03 ml/min and 0.2 ± 0.001 ml/min, respectively. The AUC of cIgG (96.9 ± 11.7 min × μg/ml) in the perfusate was 2-fold smaller than that of nIgG (196.5 ± 33.5 min × μg/ml), in which the AUC of the first phase represented 1.6 ± 1.3% of the total AUC. The hepatic extraction ratio of cIgG was 0.0085 ± 0.0007 and 2-fold higher than that of nIgG. The radioactivity of cIgG and nIgG remaining in the perfusate at the end of perfusion differed significantly, accounting for 41.1 ± 1.5% and 77.0 ± 2.9% of the total injected dose, respectively (figs. 1a and 2a). The percentage of TCA-precipitable radioactivity was always greater than 92.5% for nIgG.
The uptake of cFab by erythrocytes also declined biexponentially with an initial half-life of 4.8 ± 0.31 min. In contrast, the uptake of nFab by erythrocyte pellets was low and constant, accounting for about 7% of the total injected dose. Erythrocyte uptake of cFab, like that of cIgG, was 3-fold greater than that of nFab, and the maximal uptake was 74 ng/ml, accounting for about 30% of the total injected dose. The uptake of cFab by erythrocytes also declined biexponentially with an initial half-life of 4.8 ± 0.31 min. The measured nFab in erythrocytes was also lower, accounting for 10% of the total injected dose.

**Liver Viability.** There was no liver swelling, and no leak of perfusate occurred during perfusion with the cationized and native IgG and Fab. The bile flow rate was monitored throughout all the experiments. Despite a reduced extraction of bile at the end of the perfusion, the bile flow rate was maintained at the same level as that of controls. Fig. 6 presents the course of AST, ALT, LDH, and glucose in the perfusate vs time. Release of the three hepatic enzymes to the perfusate remained in the control range over the first 180 min and tended to be significantly lower during the last hour of the experiment. The glucose level remained constant throughout the 240 min for control and antibody liver perfusion experiments. All these data indicate that there was no liver cell lysis and no breakdown of glycogen after perfusion with cIgG and cFab.

**Antibody Uptake by PCs and NPCs.** The uptake of cIgG and cFab by both PCs and NPCs was greater than that of their counterparts nIgG and nFab, as shown in fig. 7. The uptake of cIgG by both PCs and NPCs was dose-dependent and saturable by increasing concentrations of unlabeled cIgG. The maximal uptake of cIgG did not differ significantly between PCs and NPCs at the concentration of 20 μg/ml. The uptake of nIgG by both PCs and NPCs was 6-fold lower than that of its cationized counterpart and was nonsaturable (fig. 7a).

The uptake kinetics of cFab by both PCs and NPCs were parallel (fig. 7b). The uptake profile also indicated a dose-dependent interaction, and uptake of nFab was about 8-fold lower than that of its cationized counterpart for both PCs and NPCs at the concentration of 20 μg/ml.

**Discussion**

Previous pharmacokinetic studies have shown that cationization of protein changes its pharmacokinetic properties by increasing the distribution volume and total body clearance (Pardridge et al., 1995). As a consequence, important interactions between cationized proteins and tissue could occur in major organs of clearance such as the liver and kidney. In the present experiment, biological active goat polyclonal anti-colchicine antibodies (cationized and native form) were studied using the IPR model, which allowed for physiological investigation of metabolic and biliary liver clearance and also for toxicity studies.

An in vitro model of isolated rat liver cells (PCs and NPCs) was used.
to study the interaction of cationized antibodies with these isolated liver cells. As an initial step, we checked that the immunoreactive properties of the antibodies were not altered by the cationization process. After cationization, the protein pI was raised by replacing anionic side chain carboxylic groups with aminoethylamide groups. Pardridge et al. (1994) reported that the cationization reaction might cause a decrease in the antigen binding properties of the antibody. To avoid this, cationization must be performed following preincubation of the native antibodies with the specific antigen to preserve the antigen binding capacity of the antibody during the cationization process. In our experiment, we cationized the polyclonal antibody and its Fab fragments without protection of the antigen binding sites; however, no alteration of antibody immunoreactivity was observed in terms of affinity and specificity.

Previous studies using IPRL reported a low hepatic uptake of native antibodies. For example, Cohen et al. (1962) showed a catabolic rate of 0.44%/hr for rat $^{125}$I-labeled $\gamma$-globulin; Sands and Jones (1987) found that 0.65 ± 0.17%/g of the injected dose of a murine $^{125}$I-B-3 antibody was bound to the liver at 2 hr. Early in vivo studies with radiolabeled homologous IgGs showed that hepatic uptake accounted for 8–12% of the injected dose (Beatty et al., 1990), 8–9% for the IgG subclass, and 3% for IgG$\gamma_{M}$ and 2.5 days after administration in the rat, respectively (Fukumoto and Brandon, 1981). Jones et al. (1990) showed that only 0.23 ± 0.06% of the injected dose was found in the liver 1 hr after injection of a native murine antibody in the rat. The low hepatic uptake of antibodies was also confirmed by studies of the interaction of antibodies or their fragments with the two main hepatic cell types. Neither parenchymal cells nor nonparenchymal cells showed specific uptake of IgG or Fab fragments (Bazin-Redureau et al., 1995).

We have shown that the behavior of the cationized IgG and Fab in the perfusate and erythrocyte pellet was pharmacokinetically different from that of the native IgG and Fab. Both cIgG and cFab could be fitted to a biexponential decline curve. The short initial phase probably corresponded to the rapid uptake of cIgG and cFab from the perfusate by liver cells and red blood cells. This explanation is supported by the presence of radioactivity in the perfusate erythrocyte pellets, the radioactivity being higher in the experiments with cIgG and cFab than with nlgG and nFab. Indeed, for cIgG and cFab, the initial uptake by erythrocyte pellets was 24 ± 1.21% and 30 ± 2.35%, respectively, of the total injected dose in the first 3 min. This was followed by a biexponential decline in parallel with the perfusate kinetics. In contrast, the amount of nlgG and nFab taken up by RBC was only 7 ± 0.98% and 10 ± 1.14%, respectively, of the total injected dose in the first 3 min and remained at a relatively constant level. No interaction was observed between the bovine serum albumin added into the composition of the perfusate, suggesting that the
Each value represents the mean ± SE (N = 4).

cationized antibodies can interact more with the negative charge of the cell surface membrane than with negatively charged proteins such as albumin.

Furthermore, the shorter terminal half-lives of both cationized antibodies compared with those of their native forms in the perfusate might be explained by the more marked interactions between cationized antibodies and liver cells. The absorptive-mediated interaction of the cationized antibodies with liver and RBC cells was consistent with a significantly greater volume of distribution of cIgG and cFab, which was, respectively, 52 ± 3.4% and 54 ± 3.89% greater than for the corresponding native antibodies, and by the increase in hepatic clearance and protein extraction ratio of the cationized cIgG and cFab.

The more pronounced interactions between liver cells and cationized antibodies could explain why deiodination was more elevated with both cationized antibodies than with the native forms. The degradation of radiolabeled antibodies during perfusion was measured by TCA precipitation. After 3 hr of perfusion, the TCA-precipitable percentage of nIgG and nFab remained almost constant. However, the TCA-precipitable percentage of cIgG and cFab decreased gradually over time. At the end of perfusion, only about 60 ± 5.81% and 70 ± 6.42% of cIgG and cFab were TCA-precipitable (figs. 1 and 3). This decrease could be associated with the degradation of the cationized IgG and Fab. Previous in vitro studies had reported similar results, in which the TCA-precipitable plasma radioactivity of the ^125I-cationized human immunoglobulin decreased from 92 ± 1.8% to 24 ± 3% in 6 hr (Pardridge et al., 1996).

After cationization, the antibody molecules were more positively charged, which allows their adhesion to the slightly anionic cell membrane followed by endocytosis. Our data are in agreement with early reports. Smith and Borchardt (1989) studied the binding mechanism of cationized albumin using primary cultures of bovine brain capillary endothelial cells and found that the binding and transcellular transport of cationized albumin seemed to proceed by an absorptive-phase endocytic mechanism; Triguero et al. (1991) confirmed that cationization of immunoglobulin G resulted in enhanced organ uptake of the protein after iv administration in rats and primates; Pardridge et al. (1994 and 1995) showed that cationization of a monoclonal antibody to the human immunodeficiency virus REV protein also enhances cellular uptake.

This in vitro study allowing the isolation of the two main hepatic cells provides evidence for a saturable and dose-dependent uptake of cIgG and cFab by isolated PCs and NPCs. This uptake might represent the combined processes of binding and endocytosis in vitro (Pardridge, 1986). The nonsignificant difference (p > 0.05) in the uptake of cIgG by PCs and NPCs (mainly endothelial and Kupffer cells) and the nonsignificant difference (p > 0.05) in the uptake of cFab by PCs and NPCs suggest that the mechanism by which cIgG and cFab gain access to PCs or NPCs might involve the interaction of the positive charge of the cationized antibodies with the negative charge on the surface of these two types of liver cells. The higher uptake of the cationized antibodies than their native forms by red blood cells may also support our hypothesis. Such an uptake mechanism has already been reported in studies of cationized [3H]albumin by interaction with brain capillaries in vitro (Kumagai et al., 1987).

The more intense interaction in the liver raises the issue of toxicity of cationized antibodies. A slight increase in enzyme levels was observed with increasing perfusion time in the liver perfused with cIgG and cFab, but this increase was less than in the control group. As shown under Results, the release of ALT, LDH, and AST into the perfusate with cIgG or cFab was significantly less than in the control group after 180 min of perfusion. This result might suggest that cationized antibodies bind to the surface of hepatocytes and may also penetrate into the cells by electrostatic interaction. This interaction might exert the function to inhibit the release of enzymes or protect against the lysis of cytosols. The result may illustrate that liver perfused with cIgG or cFab under our experimental conditions does not lead to release of these enzymatic proteins from cell cytosol.

The hepatotoxicity of cIgG and cFab was also investigated in vitro after the incubation with PCs and NPCs for 2 hr at 37°C. No morphologic changes in PCs and NPCs were observed, and cell viability was about 95% as determined by trypan blue exclusion and 90% by measurement of LDH release into the medium.

In conclusion, 1) cationization enhanced liver and erythrocyte uptake of both cIgG and cFab in IPRL, 2) enhanced but similar hepatic uptake of cIgG and cFab was also observed in isolated rat liver cells including parenchymal and nonparenchymal cells, and 3) neither cationized nor native antibodies exhibited tissue toxicity in IPRL or in isolated liver cells.

Acknowledgments. The technical assistance of Mrs. S. Martin, V. Piquet, and N. Taslau is gratefully acknowledged.

References


Fraker PJ and Speck JC (1978) Protein and cell membrane iodination with a sparingly soluble chloroamide, 1,3,4,6-tetrachloro-3a,6a-diphenylglycoluril. Biochem Biophys Res Commun 80:849–857.

