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研究報告の概要

＜背景＞輸血によるTSE感染のリスクはプリオンたん白の病理学的イソフォーム（PrP^{sc}）の血中濃度に依存するが、細胞PrP（PrP^{c}）の血中濃度によっても影響を受ける可能性がある。これらの濃度はPrPの血液クリアランスによって制御されるが、これについてはこれまで評価されたことはなかった。
＜研究デザイン及び方法＞ヒツジの精製された原核生物の組み換えPrP（rPrP）の血液(実際は血漿)クリアランスは、遺伝子型の異なるヒツジおよび腎摘出されたヒツジを用いて測定した。スクレイピー関連のフィブリルの静注後のプロテイナーゼK抵抗性PrP断片(PrP^{res})への曝露についても、ヒツジで調査した。
＜結果＞rPrPのARR変異型は、VRQ変異型よりもより早く除去された。感受性の高いホモ接合体のVRQヒツジのPrP^{c}血漿濃度は、ホモ接合体のARR抵抗性ヒツジのそれよりも大きく、PrP^{c}のARR変異型のクリアランスがVRQ変異型のそれよりも大きいことを示唆している。rPrPの血漿クリアランスは、両方の腎臓摘出後は52%減少し、このことはrPrP除去において腎臓が重要な寄与をしていることを示している。PrP^{res}は、スクレイピー関連断片の静注後はゆっくりと除去されることが判明した。
＜結論＞PrP宿主の遺伝型及び生理病理学的要因は、血液のPrPクリアランスを調節することでTSE感染リスクに影響する。このリスクは、静注後にPrP^{res}へ曝露が続くことによって増大する。投与された物質は実際の種と異なるが、これらはPrPクリアランスのメカニズムを調査するためのプローブとして重要である。

報告企業の意見	今後の対応
ヒツジに静注したプリオンたん白の血液クリアランスは、宿主の遺伝子的及び生理病理学的要素に影響を受けるとの報告である。 これまで血漿分画製剤によってvCJD、スクレイピー及びCWDを含むプリオン病が伝播したとの報告はない。しかしながら、万一vCJD感染者の血漿が本剤の原料に混入した場合には、製造工程においてプリオンを低減し得るとの報告があるものの、製剤から伝播する可能性を完全には否定し得ない。そのため、弊社の血漿分画製剤の製造工程におけるTSE感染性低減に関する検証実験を加速し、自社データを早期に取得し、工程評価を行い、必要に応じて工程改善を実施する予定である。	本報告は本剤の安全性に影響を与えないと考えるので、特段の措置はとらない。

使用上の注意記載状況・その他参考事項等

代表としてノイアート（献血）の記載を示す。
2. 重要な基本的注意
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2) 現在までに本剤の投与により変異型クロイツフェルト・ヤコブ病（vCJD）等が伝播したとの報告はない。しかしながら、製造工程において異常プリオンを低減し得るとの報告があるものの、理論的なvCJD等の伝播のリスクを完全には排除できないので、投与の際には患者への説明を十分行い、治療上の必要性を十分検討の上投与すること。

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Blood clearance of the prion protein introduced by intravenous route in sheep is influenced by host genetic and physiopathologic factors

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BACKGROUND: The risk of transmissible spongiform encephalopathy (TSE) transmission by blood transfusion is dependent on the blood concentrations of the pathologic isoform of prion protein (PrPsc) but may also be influenced by blood concentrations of cellular PrP (PrPc). These concentrations are controlled by the blood clearance of PrP, which has never been evaluated.

STUDY DESIGN AND METHODS: The blood (actually plasma) clearance of ovine purified prokaryote recombinant PrP (rPrP) was measured in genotyped and in nephrectomized sheep. The exposure to proteinase K-resistant fragments of PrP (PrPres) after intravenous (IV) administration of scrapie-associated fibrils (SAFs) was also investigated in a sheep.

RESULTS: The ARR variant of rPrP was eliminated more rapidly than its VRQ counterpart. The PrPc plasma concentrations in homozygous highly susceptible VRQ sheep were greater than in homozygous ARR-resistant sheep, suggesting that clearance of the ARR variant of PrPc was higher than that of the VRQ variant. The plasma clearance of rPrP was decreased by 52 percent after a bilateral nephrectomy indicating the significant contribution of the kidneys in eliminating rPrP. PrPres was shown to be slowly eliminated after IV administration of scrapie-associated fibrils.

CONCLUSION: PrP host genotype and physiopathologic factors could influence the risk of TSE transmission by modulating blood PrP clearance. This risk was increased by the sustained exposure to PrPres after IV administration. It should be noted that although the materials that have been administered (rPrP and SAFs) were not the actual species of interest, they can be of value as probes for investigating PrP clearance mechanisms.

Transmissible spongiform encephalopathies (TSEs) are neurodegenerative diseases that occur in humans (e.g., Creutzfeldt-Jakob disease [CJD]) and animals including sheep (scrapie) and cattle (bovine spongiform encephalopathy [BSE]). These diseases are characterized by the cerebral deposition of the pathologic isoform (PrPsc) of a host-encoded cellular prion protein (PrPc) that is highly expressed in the brain.

Attempts to detect infectivity in the blood of animals naturally affected with TSE have often been inconclusive.[1,2] PrPsc or infectivity, however, has been evidenced in blood from intracerebrally inoculated rodents[3] and in

ABBREVIATIONS: MRT = mean residence time(s); Plgn = plasminogen; PrP = prion protein; PrPc = cellular PrP; PrPres = proteinase K-resistant fragment of PrP; PrPsc = pathologic isoform of PrP; rPrP = recombinant PrP; SAF(s) = scrapie-affected fibril(s); TSE(s) = transmissible spongiform encephalopathy(-ies).

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blood of sheep naturally[4] or experimentally infected with scrapie[5] or BSE.[4,6]

Evidence that variant CJD (vCJD) is transmitted by transfusion has accumulated, initially from animal models, including primates, in which the high efficiency of the intravenous (IV) route of infection for BSE transmission has been demonstrated.[7] There is no longer any doubt concerning the risk of blood products obtained from individuals during the presymptomatic phase of vCJD. A third case has been reported of autopsy-confirmed vCJD infection (and a second case of clinical vCJD) from a cohort of recipients who had received transfused blood products from individuals subsequently diagnosed with vCJD.[8] More recently, a fourth symptomatic case of vCJD infection has been identified in a patient who received a transfusion from the same donor as the third case.[9] This fourth incidence considerably strengthens the evidence for prion transmission by transfusion.[10]

The pathogenesis of TSE disease highlights the importance of the PrPc as a substrate for PrPsc replication in tissue. Hence, the risk of TSE transmission by blood transfusion could be affected by the level of blood PrPc. This is controlled by its own blood clearance and modified by factors affecting this variable. The transfusion risk will also depend on the ability of the body to clear the abnormal prion protein (PrPsc), that is, the plasma (blood) clearance. This clearance variable, essential to the estimation of systemic exposure, has never been evaluated.

The aim of this study was to document the exposure of sheep to the recombinant prion protein (rPrP) after IV administration and to identify genetic and pathophysiologic factors that might modulate such exposure. Sheep were chosen because of the similarity of the pathogenesis of scrapie with vCJD and because the impact of Prnp gene polymorphism on the susceptibility to scrapie infection is well documented. The $V_{136}R_{154}Q_{171}$ (VRQ) and ARR alleles have consistently been associated with high susceptibility and natural resistance to the clinical disease, respectively,[11-13] although atypical scrapie strain(s) can naturally infect sheep harboring the so-called resistant PrP genotype.[14] The ARR and VRQ genetic variants of the purified prokaryote rPrP were used as probes and only as probes (see Discussion) to test the hypothesis that the higher intrinsic stability of the VRQ variant relative to its ARR counterpart[15,16] could result in a lower in vivo clearance rate of the VRQ protein.

This approach also allowed us to examine the hypothesis that the subject's genetic background could directly influence PrP clearance and to evaluate the contribution of the kidneys to the overall clearance of plasma PrP, the kidney being a major organ for protein clearance.[17] The fate of ovine proteinase K-resistant fragment of PrP (PrPres) during the first hours after its direct entry in the blood was also evaluated in one sheep to assess the ability of the body to clear the scrapie agent.

MATERIALS AND METHODS

General

All experimental procedures were performed in accordance with French legal requirements regarding the protection of laboratory animals and under authorization number 31242 from the French Ministry of Agriculture.

Design

The objectives of Experiment 1 were 1) to compare the pharmacokinetics of the VRQ and ARR genetic variants of the purified prokaryote ovine rPrP; 2) to examine the influence of the genotype of the test animal on plasma pharmacokinetic variables of rPrP; and 3) to test the hypothesis of a first pass effect at the level of the brain, that is, direct trapping of the protein during its initial transit across the head.

The experiment was performed on eight healthy Romanov ewes: four homozygous VRQ at codons 136, 154, and 171 of the Prnp gene and four homozygous ARR. The VRQ ewes were 1 year old and weighed 42.1 ± 2.7 kg and the ARR ewes were 2 years old and weighed 51.2 ± 5.5 kg. The experiment involved two periods separated by 2 days. During the two periods, the ewes received an IV administration of rPrP (VRQ vs. ARR, 0.02 mg/kg) according to a crossover design. Ten days later, one ARR and two VRQ ewes received an intraarterial (external carotid) administration of the VRQ variant (0.02 mg/kg) to compare the pharmacokinetic variables of rPrP after arterial and IV administrations.

The ex vivo stability of the variants of rPrP in blood and plasma was compared by separately adding 145 ng of each of the ARR and VRQ variant of rPrP to 10-mL aliquots of fresh sheep blood and plasma that were incubated at 37°C under constant stirring. Samples were taken at 0.5, 1, 2, 3, 4, 5, 6, and 24 hours. The plasma was immediately separated from blood after centrifugation for 10 minutes at 1400 × *g* and all the plasma samples were stored at –20°C until PrP assay.

Experiment 2 was designed to evaluate the role of the kidneys in the clearance of rPrP and to examine the fate of PrPres after its direct entry into the blood. The first part of the experiment was performed with three Lacaune ewes aged from 5 to 8 years and weighing 38 to 55 kg. The pharmacokinetic variables of the VRQ variant of the rPrP intravenously administered (0.02 mg/kg) were determined before (control period) and immediately after a bilateral nephrectomy (experimental period). During the control period, one ewe was anesthetized according to the same protocol as that used for surgery. During the experimental period, which took place 1 to 14 days later, anesthesia was induced with sodium thiopental (Nesdonal, Merial, Lyon, France; 20 mg/kg) and maintained for 24 hours by repeated administrations of 0.2 to 0.3 mg per kg sodium

thiopental at 20- to 30-minute intervals. The ewes were bilaterally nephrectomized according to the method previously described.[18] Immediately after surgery, three control blood samples were obtained at 20-minute intervals and the VRQ variant was intravenously administered. The ewes were sacrificed in extremis. The second part of the experiment was performed with one young Lacaune ewe aged 6 months and weighing 30.5 kg that received an IV administration of 125 mL of scrapie-affected fibrils (SAFs) in 0.8 mol per L urea. The quantity of PrPres administered was evaluated at 75 µg equivalent of VRQ rPrP.

Ten-milliliter blood samples were collected at 20-minute intervals for 1 hour before administration; at 1, 2, 4, 8, 15, 30, 45, 60, 90, and 120 minutes after the variant administration; at 1-hour intervals for 12 hours; and finally at 24, 36, and 48 hours after administration, for Experiment 1 and the first part of Experiment 2, respectively. The same protocol was used for the second part of Experiment 2 except that 15-mL blood samples were also obtained every day until Day 7 after SAF administration.

For all experiments, in intact ewes, all the urine in the bladder was removed before the administrations and then at 1-hour intervals for 12 hours and at 3-hour intervals during the following 12 hours. The total volume of urine removed was measured in each case.

Brain extraction

Brain samples were obtained from three Romanov homozygous VRQ ewes naturally affected with scrapie. The frozen brain samples were homogenized to give a 20 percent (wt/vol) suspension in buffer (Bio-Rad, Marnes la Coquette, France) and PrPres was extracted with the purification protocol of the Bio-Rad TSE test (TeSeE sheep/goat purification kit, Bio-Rad) except that the final precipitate was solubilized with 4 mol per L urea (Sigma-Aldrich, Lisle d'Abeau Chesnes, France) and stored at –20°C until administration. The extract was diluted five-fold in 0.1 mol per L phosphate buffer containing 1 mg per mL bovine serum albumin (BSA) and 0.15 mol per L NaCl (Sigma-Aldrich), heated at 100°C for 5 minutes, and sonicated for 15 minutes at 560-W power setting (Transsonic 95HL, Prolabo, Fontenay sous Bois, France) in the hour preceding the administration. The PrPres content of the solution was measured by enzyme-linked immunosorbent assay (ELISA). Seventy-five micrograms of PrPres (equivalent to VRQ rPrP) was obtained from 100 g of brain tissue.

Administration and sampling

The IV administrations were performed in the right jugular vein via an indwelling catheter (Hemocath, Vygon, Ecouen, France). The intraarterial administrations were performed in the right external carotid artery via an intraarterial catheter (BD Careflow, Becton Dickinson, Le Pont-de-Claix, France) inserted in anesthetized ewes 2 days before the administrations. The VRQ and ARR variants of the ovine PrP were expressed in *Escherichia coli* and purified according to the method previously described.[15] Previous authors have shown that the recombinant proteins are monomeric in solution. The variants were kept at 4°C in solution in 20 mmol/L MOPS (Sigma-Aldrich), pH 7.25, at a concentration of approximately 1 mg per mL. The protein concentration was measured from the optical density at 280 nm with the extinction coefficient of 58718.0 mol per L per cm. ARR and VRQ solutions of rPrP were prepared in sheep plasma at a concentration of 0.2 mg per mL and kept at –20°C for all administrations.

Blood samples were collected from the left jugular vein via an indwelling catheter into ethylenediaminetetraacetate-containing tubes and centrifuged for 10 minutes at 1400 × *g*. The plasma was separated and stored at –20°C until assay. Urine was obtained via an indwelling closed urethral catheter (Rüsch, Teleflex Medical, Le Faget, France) that was kept in the bladder for 24 hours. Samples were stored at –20°C until analysis.

Quantification of PrP with a two-site enzyme immunoassay

Two immunometric assays were adapted from the method previously described.[19] The plasma and urine concentrations of purified prokaryote rPrP in all samples were measured by ELISA with BAR210, an anti-N terminal monoclonal antibody (MoAb)[20] recognizing residues 26 to 34 and the 12F10-AchE Spi Bio (Massy, France) antibody, an anti-C terminal MoAb recognizing residues between amino acids 154 and 171. Native plasma PrP, which lacks the BAR210 epitopes, was monitored by ELISA in plasma samples collected before administration of the rPrP, with the SAF34 and 12F10-AchE Spi Bio antibodies. Standard curves ranging from 0.5 to 20 ng per mL were established by diluting rPrP (VRQ and ARR) in ovine plasma, urine, or enzyme immunoassay (EIA) buffer (0.1 mol/L phosphate buffer, pH 7.4, 0.15 mol/L NaCl, 0.1% BSA, NaN_3 0.01%; Sigma-Aldrich).

Plasma (or urine) rPrP concentrations were calculated from the optical readings obtained by reference to the standard curve established with plasma (or urine) supplemented with the corresponding variant. Native PrP plasma concentrations were calculated from the optical readings obtained by reference to the standard curve established with EIA buffer solutions of the variants of rPrP. As an illustration, standard curves of ovine rPrP in EIA buffer or in plasma are shown in Fig. 1.

The limit of quantification of native and rPrP assays was 0.5 ng per mL. The accuracies of the PrP assay of plasma samples supplemented with the solution of VRQ

appropriate volume to consider when determining the amount of PrP in the body at equilibrium, was obtained from

$$V_{SS} = V_C[1+(k_{12}/k_{21})],$$

where V_C is the volume of the central compartment, k_{12} is the first-order rate constant of transfer from the central compartment to the peripheral compartment, and k_{21} is the first-order rate constant of transfer from the peripheral compartment to the central compartment. The total plasma clearance, which expresses the capacity of the organism to eliminate proteins (Cl_{TOT}, mL/(kg·min)), was calculated with

$$Cl_{TOT} = \text{dose}/AUC,$$

where AUC is the area under the plasma PrP concentration–time curve obtained by integrating the equation

$$AUC = A/\alpha + B/\beta.$$

The clearance of distribution was calculated with

$$Cl_D = k_{12}V_C.$$

The appropriate volume to consider when calculating the amount of PrP remaining when the pseudodistribution equilibrium has been reached, V_{area} (mL/kg) was obtained from

$$V_{area} = Cl_{TOT}/\beta,$$

where β is the slope of the terminal phase. The terminal plasma half-life ($t_{1/2}$, min) was obtained from

$$t_{1/2} = \text{Log}2/\beta.$$

Different mean residence times (MRTs) were calculated.[24] The MRT (min), that is, the mean total time taken for each PrP molecule to transit through the body, was calculated with

$$MRT = (A/\alpha^2 + B/\beta^2)/(A/\alpha + B/\beta).$$

The MRT in the central compartment (MRT_C), that is, the mean time spent by the protein within the measured compartment was obtained from

$$MRT_C = 1/k_{10},$$

where k_{10} is the first-order rate constant of elimination from the central compartment. The MRT in the peripheral compartment (MRT_T), that is, the mean time spent by the protein outside the measured compartment, was obtained from

$$MRT_P = MRT - MRT_C.$$

Statistical analysis

Results are reported as mean ± standard deviation (SD). Statistical analyses were performed with computer software (SYSTAT 8.0, SPSS, Inc., Chicago, IL). PrP concentrations below the limit of quantification of the assay were arbitrarily fixed at 0.5 ng per mL. p Values lower than 0.05 were considered as significant. The pharmacokinetic variables of PrP genetic variants were compared between homozygous ARR and VRQ ewes with repeated-measures analysis of variance (ANOVA) with ewes as a random effect factor and the genetic variant of PrP, the ewe genotype, and their interactions as fixed effect factors. The genotype effect was tested with "ewe within-group variance" as the residual term. The mean pharmacokinetic variables of the VRQ variant of rPrP obtained after IV and intraarterial administration were compared by a paired t test. The effect of genotype on basal plasma PrPc concentrations was analyzed with repeated-measures ANOVA with ewes as a random effect factor, and genotype and time as fixed effect factors. The mean pharmacokinetic variables of the rPrP obtained before and after nephrectomy were compared by a paired t test. The effect of time on plasma PrP concentrations in vitro was analyzed by ANOVA with medium (blood vs. plasma), genetic variant, and time as fixed effect factors.

RESULTS

Pharmacokinetic variables of the prokaryote rPrP

Figure 2 shows, in a representative ewe, the semilogarithmic plots of the ARR and VRQ variants of the purified prokaryote rPrP after IV administration (0.02 mg/kg).

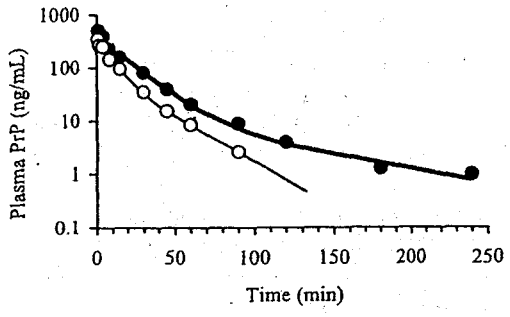

Fig. 2. Representative plasma concentrations of the genetic variants of PrP after IV administration. The observed ARR (○), VRQ (●), and corresponding fitted ARR (thin line) and VRQ (thick line) plasma concentrations were obtained in a representative homozygous VRQ sheep that received IV administrations of the ARR and VRQ genetic variants of the purified ovine prokaryote rPrP at a dose of 0.02 mg per kg.

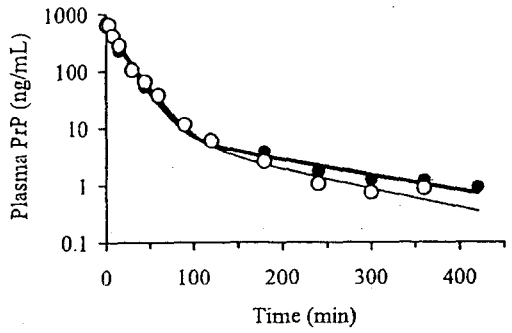

Fig. 4. Representative plasma concentrations of PrP according to its route of administration. The observed (●) and corresponding fitted (thick line) plasma concentrations of the VRQ variant of the purified ovine prokaryote rPrP were obtained after IV administration of the variant (jugular vein) at 0.02 mg per kg and the observed (○) and corresponding fitted (thin line) plasma concentrations of the VRQ variant were obtained after its administration at the same dose by the intraarterial route (external carotid) in a representative ARR sheep.

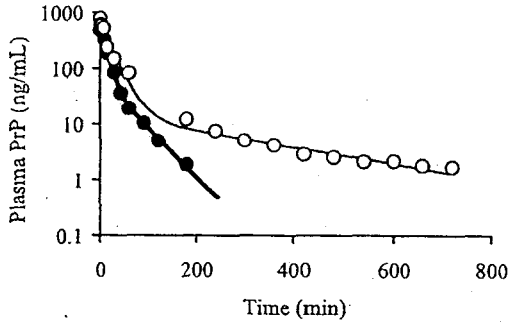

Fig. 5. Representative plasma concentrations of PrP after its administration before and after nephrectomy. The symbols represent the observed plasma concentrations of VRQ variant of the purified ovine rPrP obtained before (●) and after (○) a bilateral nephrectomy in a representative sheep intravenously administered with the VRQ variant at 0.02 mg per kg. The lines represent the corresponding fitted plasma concentrations of the VRQ variant obtained before (thick line) and after (thin line) nephrectomy.

TABLE 2. Pharmacokinetic variables (mean ± SD) of the VRQ variant of the purified prokaryote rPrP according to the route of administration*

Variables	Route of administration	
	IV	Intraarterial
Cl_{TOT}, mL/(kg·min)	1.98 ± 0.41	2.41 ± 0.90
$t_{1/2}$, min	85.8 ± 28.3	68.4 ± 18.8
V_C, min	40.6 ± 9.10	47.8 ± 12.2
V_{SS}, mL/kg	74.0 ± 26.7	72.4 ± 26.3
V_{area}, mL/kg	233.2 ± 30.1	227.6 ± 359.3
MRT, min	37.9 ± 12.9	30.8 ± 8.70
MRT_C, min	20.5 ± 1.48	19.5 ± 2.50
MRT_P, min	17.4 ± 11.5	11.3 ± 7.10

* The pharmacokinetic variables were obtained after an IV (jugular vein) or an intraarterial (external carotid) administration of the VRQ variant at a dose of 0.02 mg per kg to three sheep. Abbreviations are explained in Table 1.

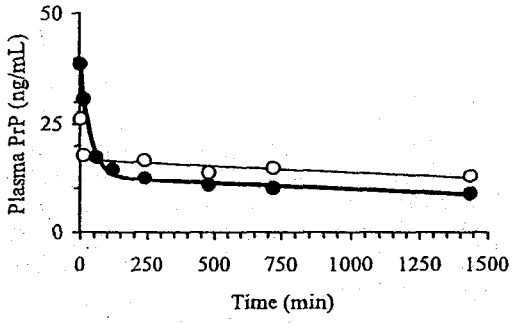

Fig. 6. Plasma PrPres concentrations after administration of SAF solution. The symbols represent the observed plasma concentrations of PrPres obtained with the PIgn-coated beads extraction method (○) or after a 60°C heat treatment (●) in a sheep intravenously administered an SAF solution containing a 75-µg equivalent of VRQ prokaryote rPrP. The lines represent the corresponding fitted plasma concentrations measured by the PIgn-coated beads method (thin line) and after the 60°C heat treatment (thick line).

Mechanisms of plasma PrP clearance

Figure 5 shows the temporal variations in plasma concentrations of the VRQ variant of rPrP after IV administration of 0.02 mg per kg to a representative sheep before and after a bilateral nephrectomy. Figure 5 shows the dramatic effect of nephrectomy, with a much slower elimination rate in the nephrectomized sheep. The mean plasma clearance of the VRQ variant was 2.1 times lower after bilateral nephrectomy (1.56 ± 0.66 mL/(kg·min) vs. 0.75 ± 0.36 mL/(kg·min); t test, $p < 0.05$). The renal PrP clearance derived from plasma clearance values obtained before and after nephrectomy (0.81 ± 0.30 mL/(kg·min)) represented 52 percent of the total clearance. The mean residence time of the protein was 2.6-fold higher (24.2 ± 7.6 min vs. 65.2 ± 2.6 min; $p < 0.05$, t test) after nephrectomy. The mean value of the volume of the central compartment obtained after nephrectomy was slightly lower than that obtained during the control period but this difference was not relevant (22.3 ± 9.82 mL/kg vs. 24.0 ± 9.69 mL/kg; t test, $p < 0.01$). No other pharmacokinetic variables were affected by nephrectomy.

Figure 6 shows the temporal decrease in plasma PrPres concentrations after the IV administration of an SAF solution containing 75 µg equivalent of VRQ rPrP. After a relatively fast distribution phase, the plasma PrPres concentrations decreased very slowly and reached the detection limit of the assay (7.5 ng/mL) 24 hours after administration. The estimated plasma clearance of PrPres

was 0.24 or 0.43 mL per (kg·min) based on the results obtained after Plgn-coated microbead capture or 60°C heating, respectively. The estimated mean terminal half-lives obtained after Plgn-coated microbead capture or by the 60°C heating method were 58 and 41 hours, respectively. The mean residence time and the steady-state distribution volumes were 5030 minutes (84 hr) versus 3435 minutes (57 hr) and 1187.0 versus 1487.4 mL per kg, respectively (Plgn-coated microbead capture method vs. 60°C plasma heating treatment). If the results obtained by Plgn-coated microbead capture are considered, then the MRT_C and MRT_P values are equivalent. PrPres could not be detected in any of the urine samples collected after administration of the SAF solution.

DISCUSSION

Before any discussion of the present experiments, we must acknowledge that we tested our different hypotheses with a rPrP of bacterial origin and not the native form of the PrP, which is not currently available for pharmacokinetic studies. The natural form is glycosylated and the glycosylation of a protein may considerably influence its fate in the body. We nevertheless believe that this pioneer work may have some value to explore the clearance pathways and the influence of the genotype and the role mediated by the conformation of the variant of PrP and/or by the clearance mechanisms. Similarly, the disease-associated isoform of PrP that was administered underwent a series of physicochemical processes before its administration (solubilization) that were able to partly denature the protein. Thus, the different test proteins used in the present experiment should be considered only as probes. We hypothesized they were able to explore, at least qualitatively, and possibly quantitatively (i.e., giving an order of magnitude), different disposition processes of physiologic interest, mainly concerning the overall plasma clearance that contributes to the systemic exposure.

Our results show that clearance of the rPrP is low and of the same order of magnitude as the glomerular filtration rate in sheep[25] (approx. 2 mL/(kg·min)). Clearance of the ARR variant of rPrP associated with resistance to scrapie was almost twice that of the VRQ variant associated with scrapie susceptibility. For a given variant, however, the genotype of the recipient had no effect on PrP plasma clearance, indicating that the protein conformation, not the animal's clearance mechanisms, was responsible for the difference between the two genotypes.

It is generally accepted that the kidney is the major organ of protein elimination[26] and this was confirmed in the present experiment where the kidney clearance of the VRQ variant of rPrP was about half the total clearance. Despite the high renal clearance, no PrP was found in urine. This is not really surprising because most of the low-molecular-weight proteins (here approx. 23 kDa for our variants) that are filtered through the glomerulus are metabolized by enzymes located in the brush border of the tubular lumen.[26] We did not investigate the renal clearance of the ARR variant. It is generally accepted, however, that the renal excretion of proteins is mainly governed by their size and that the sieving effect of glomerular filtration is independent, for small proteins, of molecular charge[27] or other conformational differences. Because ARR and VRQ are of the same molecular weight, it can be hypothesized that the observed difference in clearance of the ARR and VRQ recombinant proteins was of nonrenal origin.

Nonrenal clearance of rPrP was partly explored in our experiment and the direct metabolism of rPrP by plasma proteases can be excluded because we showed that the plasma concentrations obtained from blood or plasma supplemented with either the VRQ or the ARR variant and incubated at 37°C did not vary over a 6-hour period, that is, a time greater than that required to observe PrP elimination in vivo. Similarly, we can exclude a selective trapping of rPrP in the brain because there was no evidence of a first-pass effect when the recombinant protein was directly administered through the carotid artery. It is likely that, as for many other proteins, the main nonrenal clearance mechanism involves the reticuloendothelial system. The liver was shown to contribute significantly to protein metabolism, especially through receptor-mediated endocytosis followed by degradation in lysosomes. This mechanism, contrary to the bulk filtration of protein at the glomerular level, is likely to be a more specific process of protein elimination that could explain the difference in clearance that we observed between the two tested variants.

It is generally accepted that proteins are initially distributed into the plasma volume and then more slowly into the interstitial fluid space. This view is supported by the present experiment where the initial distribution volume of rPrP approximated to that of the plasma volume (overall mean $V_C = 43$ mL/kg); in contrast, the volume of distribution associated with the terminal disposition phase of rPrP was of the same order of magnitude as the volume of extracellular fluid ($V_{area} = 162$ mL/kg), indicating that at least a fraction of the administered rPrP gained access to the extracellular fluid. Owing to this restricted distribution, the overall mean residence time of the recombinant protein was rather short, ranging from 14.5 to 52 minutes, despite the low clearance rate. The greater mean residence time of the VRQ variant, when compared with that of the ARR variant, was mainly explained by the greater mean residence time of the VRQ variant in the central compartment as measured by the MRT_C (20 min vs. 12.5 min). By contrast the MRT_P (i.e., the overall MRT of the protein in the peripheral compart-

ment) was relatively similar for the two variants (8 min vs. 11 min).

These results obtained with the nonglycosylated purified prokaryote recombinant protein may provide useful information for plasma PrPc because some authors[28-33] have shown that the three-dimensional structure and the thermal stability of rPrP produced in *E. coli* are essentially identical to those of the natural glycoprotein. Thus, as expected, the glycosylation and the GPI anchor did not affect the folding of the PrP protein and transgenic mice expressing a nonglycosylated PrPc as well as transgenic mice harboring a PrP without a GPI anchor[34,35] are able to replicate the infectious agent. Furthermore, we have shown that the basal plasma PrPc concentrations of homozygous VRQ ewes were almost twice those of homozygous ARR ewes. This suggests that the plasma clearance of the ARR variant of PrPc is, like the same variant of rPrP, nearly twice that of its VRQ counterpart assuming that there is no difference in the synthesis of the protein and that rPrP may be a relevant probe for studying the fate of PrPc. Our results are in agreement with those of Halliday and coworkers[36] who showed that the level of PrPc expressed on the cell surface of peripheral blood mononuclear cells was influenced by the genotype, with the highest levels found in scrapie-susceptible homozygous VRQ sheep and the lowest in scrapie-resistant homozygous sheep. The level of PrP expression by blood cells was correlated with the level of ovine plasma PrPc by Thackray and colleagues[37] who showed genotypic differences in the level of ovine plasma PrPc, with the highest and lowest levels being observed in plasma from homozygous VRQ and ARR sheep, respectively. The higher plasma PrPc concentrations of dogs affected by renal insufficiency when compared to healthy ones (unpublished observations) together with previous observations in humans with extensive renal insufficiency[38,39] strongly suggest that the kidneys contribute highly to PrPc clearance.

Our results raise the question of the significance of host genetic and pathophysiologic (renal insufficiency)-caused variations of plasma PrPc levels with respect to TSE susceptibility. The implication of PrPc plasma levels for peripheral pathogenesis of scrapie is still debated because blood transmissions can occur in species such as hamsters where blood levels of normal PrP are exceedingly low. It cannot be ruled out, however, that some knowledge of the influence of genetic and pathophysiologic factors on plasma concentrations or clearance of the normal protein provides a ground for future investigations aimed at a better understanding of the role of plasma PrPc in the transmissibility of the infectious agent by the IV route.

For the single sheep that we investigated, the kinetics of the temporal decrease in plasma PrPres concentrations after the IV administration indicated that the disease-associated isoform of PrP (clearance of 0.24 mL/(kg·min)) is eliminated much more slowly than the recombinant genetic variants of PrP (2-3 mL/(kg·min)), resulting in a greater mean residence time (approx. 84 hr vs. 14-52 min). In addition, we observed a high steady-state volume of distribution for PrPres (1.2-1.5 L/kg) suggesting that PrPres is more widely distributed than rPrP. It should be stressed that our pharmacokinetic approach required the solubilization of PrPres recovered as pellets though we cannot exclude the persistence of insoluble aggregated forms of PrPres in the administered SAF preparation despite the ultrasonic and heating treatments. Despite such a limitation, the difference in clearance between the recombinant and pathologic isoforms of PrP is so great that we have no doubt that the disease associated isoform of PrP is eliminated much more slowly than the recombinant protein. The limit of our analytical method prevented us from evaluating PrPres concentrations below the level of quantification (7.5 ng/mL) and from ensuring that PrPres did not persist for a longer time at low concentrations.

Considerable uncertainty exists about the relevant spiking form of prion to document the risk of TSE transmission by blood transfusion.[40] Data from a rodent experimental model of TSE suggest that the infectious agent in plasma is very small, unsedimentable, and poorly aggregated,[41,42] but many attempts to solubilize PrPSc under nondenaturing conditions have been unsuccessful[43,44] until recently.[45] In the present clearance study, it must be assumed that the method used to prepare the prion material from sheep brain must be efficient and safe enough to obtain a PrPres dose that can be administered intravenously to a sheep. The purpose of this single PrPres infusion was no more than to obtain a first estimate of the order of magnitude of the clearance of the disease-associated form of PrP.

In conclusion, we have shown, by use of purified prokaryote rPrP, that the clearance of the ARR variant associated with resistance to scrapie is greater than the clearance of the VRQ variant associated with sensitivity to scrapie. This, together with the higher basal plasma PrPc concentrations observed in homozygous highly susceptible VRQ ewes compared with homozygous resistant ARR ewes, suggests that the ARR variant of PrPc is eliminated more rapidly than the VRQ variant. The 52 percent decrease in clearance of the prokaryote rPrP in nephrectomized ewes suggests that the kidneys contribute considerably to the elimination of the prion protein and that renal insufficiency could represent a risk factor for TSE disease transmission. The pathologic isoform of PrP was shown to be cleared very slowly from the blood, leading to sustained exposure after its direct IV administration.

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