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2003

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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。

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
(1) 略
1) 略
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。

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。

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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

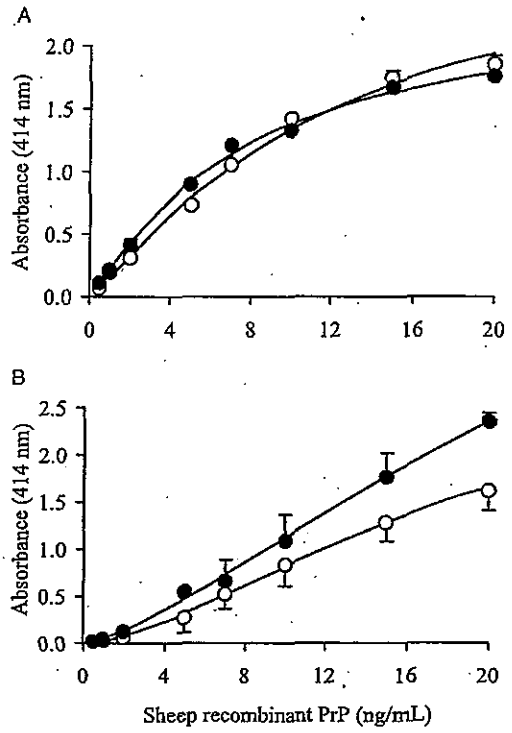

Fig. 1. Immunologic detection of ovine rPrP by two-site immunometric assays. ELISA plates were coated with capture antibodies SAF34 (A) or BAR210 (B) as described under Materials and Methods. Ovine recombinant ARR (○) or VRQ (●) variants in buffer (A) or in plasma (B) were detected by 12F10-AchE antibody. Data shown are mean ± SD of duplicate or triplicate wells. Lines represent the fitting with a non linear regression sigmoid model.

or ARR variants of rPrP at three concentrations (1.5, 6, and 12 ng/mL) were 91.1 and 94.3 percent, respectively. The mean intra- and interassay coefficients of variation for EIA buffer (plasma) solutions of the genetic variants of rPrP at these concentrations were 7.4 percent (6.5%) and 15.7 percent (13.5%), respectively.

Quantification of PrPres

Two methods were developed to measure the plasma PrPres concentrations after administration of the SAF preparation. The first method involved heating plasma at 60°C for 1 hour after diluting it by half in denaturing buffer from the TeSeE sheep/goat detection kit to denature the PrPres before assaying by ELISA. The second method was based on the natural affinity of plasminogen (Plgn) for PrPSc aggregates.[21,22] Superparamagnetic tosyl-activated M-280 microbeads (Invitrogen-Dynal, Cergy Pontoise, France) were coated with 100 µg of human Plgn (Sigma-Aldrich) in 0.1 mol per L carbonate-bicarbonate buffer, pH 9.4. The Plgn-coated microbeads were incubated at room temperature with plasma samples diluted (1:2, vol/vol) in phosphate buffer (10 mmol/L, pH 7.4) containing Sarkosyl (Sigma-Aldrich). After a 2-hour incubation with rotary shaking and three washes in 0.05 mol per L sodium phosphate buffer, pH 7.4, 1 percent Tween 20 (Sigma-Aldrich), the Plgn-coated microbeads were pelleted and heated at 105°C for 5 minutes in denaturing buffer from the TeSeE sheep/goat detection kit. The microbeads were then magnetized and discarded.

For the two methods, the supernatants and heated plasma samples were diluted fivefold in dilution buffer from the TeSeE sheep/goat detection kit before determination of plasma PrPres concentrations by ELISA (TeSeE sheep/goat detection kit). The plasma PrPres concentrations were calculated from the optical density readings obtained with reference to the standard curve established with solutions of the VRQ variant in dilution buffer and corrected for the mean extraction coefficient. The mean extraction coefficients of PrPres determined as the mean percentage of the PrPres recovered from plasma supplemented with an SAF solution (brain extract from a scrapie-affected homozygous VRQ sheep) at different levels (4, 8, and 16 mg of brain extract/mL) were 81.8 ± 21.1 and 97.6 ± 23.5 percent for the first (60°C heated plasma) and second method (Plgn-coated microbeads extraction), respectively.

Urine, half-diluted in denaturing buffer from the TeSeE sheep/goat detection kit, was heated at 105°C for 5 minutes and the PrPres concentrations were measured by ELISA with the SAF34 and 12F10-AchE Spi Bio antibodies. The extraction coefficient of PrPres, determined from urine spiked with the SAF in 0.8 mol per L urea to obtain the urinary PrPres concentration of 4 ng per mL, was 41 percent.

Kinetic analysis

Plasma PrP concentrations were analyzed with computer software (WinNonlin 5.0, Pharsight Corp., Mountain View, CA). Data were fitted to the biexponential equation

$$C(t) = A\exp(-\alpha t) + B\exp(-\beta t),$$

where A and B (ng/mL) are preexponential coefficients and α and β are exponents. The estimated variables (A, B, α, β) were used to solve the first-order rate constants of transfer from central to peripheral compartments (k_{10}, k_{12}, k_{21}) with classical equations.[23] The volume of the central compartment (mL/kg) was obtained from

$$V_C = \text{dose}/(A+B),$$

where dose is the administered dose. The steady-state volume of distribution (V_{ss}, mL/kg), which is the