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).

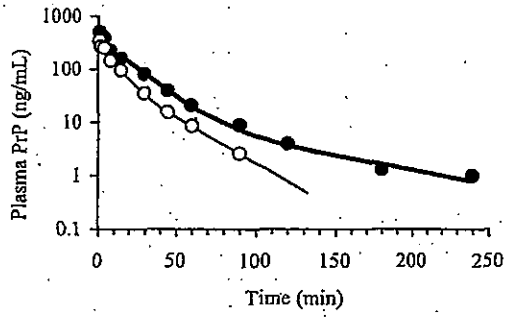

Fig. 2. Representative plasma concentrations of the genetic variants of PrP after IV administration. The observed ARR (○), VRQ (●), and corresponding fitted ARR (thin line) and VRQ (thick line) plasma concentrations were obtained in a representative homozygous VRQ sheep that received IV administrations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

Visual inspection of Fig. 2 shows that the ARR protein variant was eliminated faster than the VRQ protein. Table 1 gives the mean (±SD) pharmacokinetic variables of the VRQ and ARR variants of the rPrP intravenously administered to four healthy homozygous VRQ ewes and to four homozygous ARR ewes. Figure 3 gives a stochastic interpretation of the disposition of the ARR and VRQ variants of the rPrP. For a given genetic variant of the protein, the genotype of the recipient ewe did not influence any pharmacokinetic variables of the protein. The mean total plasma (blood) clearance (Cl_{TOT}) of the ARR variant of the rPrP was almost two times greater than that of the VRQ variant (ANOVA $p < 0.01$). The mean terminal half-life ($t_{1/2}$) of the ARR variant was nearly half that of the VRQ variant (ANOVA $p < 0.05$). The steady-state volume of distribution (V_{SS}) of the rPrP was low and was not affected by the genetic variant of the protein. The MRT of the protein was rather brief; the MRT in the central compartment (MRT_C) represented more than half the total MRT in the entire body for the two variants. The mean MRT and MRT_C of the ARR variant were lower than the values obtained for the VRQ variant (ANOVA $p < 0.01$).

TABLE 1. Mean (±SD) pharmacokinetic variables of the purified prokaryote rPrP*

	Variant		Genotype	
Variables	ARR	VRQ	ARR	VRQ
Cl_{TOT}, mL/(kg·min)	3.79 ± 0.94	2.00 ± 0.38†	2.69 ± 1.24	3.10 ± 1.11
Cl_D, mL/(kg·min)	2.99 ± 5.74	0.321 ± 0.215	2.41 ± 5.91	0.901 ± 0.917
$t_{1/2}$, min	29.9 ± 15.0	60.5 ± 27.8‡	48.9 ± 31.9	41.6 ± 22.0
V_C, min	46.4 ± 15.0	40.0 ± 9.1	42.9 ± 15.8	43.5 ± 9.12
V_{SS}, mL/kg	77.8 ± 34.2	60.9 ± 19.4	64.7 ± 28.6	74.1 ± 29.1
V_{area}, mL/kg	157.9 ± 83.6	167.3 ± 63.1	162.5 ± 91.4	162.7 ± 51.6
MRT, min	20.2 ± 6.00	30.9 ± 9.45†	25.3 ± 7.17	25.8 ± 11.8
MRT_C, min	12.5 ± 3.22	20.1 ± 3.25†	17.4 ± 5.49	15.2 ± 4.68
MRT_P, min	7.71 ± 4.77	10.77 ± 8.31	7.94 ± 4.25	10.54 ± 8.67

* The ARR and VRQ genetic variants of the purified prokaryote rPrP were intravenously administered at a dose of 0.02 mg per kg to four healthy homozygous VRQ sheep and four homozygous ARR sheep. The variant column indicates the mean (±SD) pharmacokinetic variables of the ARR and VRQ genetic variants of the rPrP while the genotype column gives the mean (±SD) pharmacokinetic variables of the two variants for the homozygous VRQ and ARR sheep, respectively.
† $p < 0.01$.
‡ $p < 0.05$.
Cl_{TOT} = total plasma (blood) clearance; Cl_D = clearance of distribution (equivalent to the clearance of redistribution, Cl_R); $t_{1/2}$ = terminal plasma half-life; V_C = volume of the central compartment; V_{SS} = steady-state volume of distribution; V_{area} = volume containing the amount of PrP remaining when the pseudodistribution equilibrium has been reached; MRT = mean residence time, that is, the mean total time taken for a PrP molecule to transit through the body; MRT_C = MRT in the central compartment, that is, the mean total time spent by the PrP molecule within the measured compartment; MRT_P = MRT in the peripheral compartment, that is, the mean time spent by the protein outside the measured compartment.

In the first experiment, PrP could not be detected in any of the urine samples collected. The mean basal plasma PrPc concentration of homozygous VRQ sheep was greater than that of homozygous ARR sheep (5.3 ± 1.2 ng/mL vs. 3.3 ± 0.7 ng/mL; ANOVA $p < 0.05$).

Figure 4 illustrates the plasma concentrations of the VRQ variant of the prokaryote rPrP after its administration at 0.02 mg per kg either by the intraarterial route or by the IV route to a representative homozygous ARR ewe. The time curve shows that both routes of administration gave very similar results. The mean pharmacokinetic variables of the VRQ variant of the rPrP obtained after intraarterial administration did not differ significantly ($p > 0.05$) from those obtained by the IV route (Table 2), thereby supporting the hypothesis of an absence of a brain first-pass effect to clear the protein.

When fresh blood or plasma samples were similarly spiked with either the ARR or the VRQ variant of the rPrP, the plasma PrP concentrations remained almost constant for incubation times ranging from 0 to 6 hours (no time effect, $p > 0.05$). After 24 hours of incubation, the mean decrease in the plasma PrP concentration ranged from 6 to 39 percent, suggesting a lack of (or very low) blood protease activity.

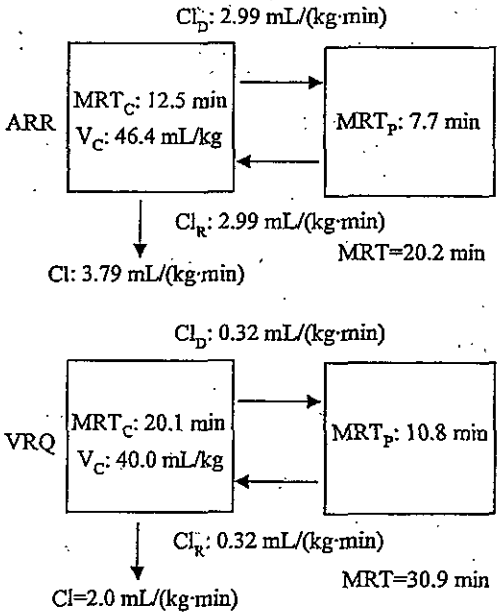

Fig. 3. Stochastic interpretation of the disposition of the ARR and VRQ variants of PrP. The values of the pharmacokinetic variables are the mean values obtained after administration of the variants of the purified ovine prokaryote rPrP by the IV route at 0.02 mg per kg to eight genotyped sheep. Abbreviations are explained in Table 1.

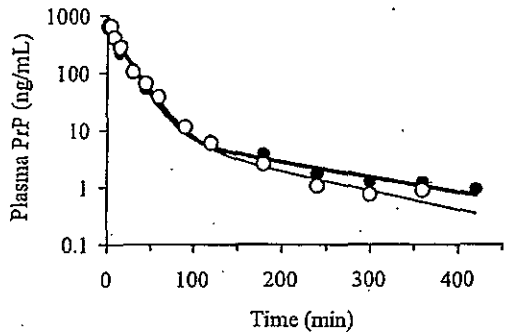

Fig. 4. Representative plasma concentrations of PrP according to its route of administration. The observed (●) and corresponding fitted (thick line) plasma concentrations of the VRQ variant of the purified ovine prokaryote rPrP were obtained after IV administration of the variant (jugular vein) at 0.02 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.

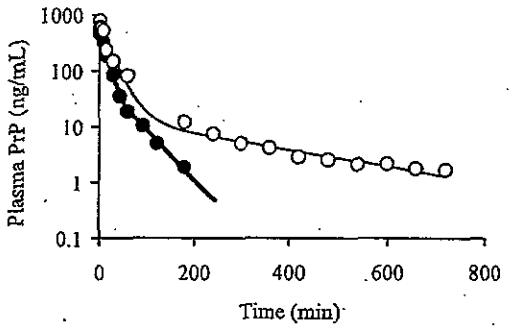

Fig. 5. Representative plasma concentrations of PrP after its administration before and after nephrectomy. The symbols represent the observed plasma concentrations of VRQ variant of the purified ovine rPrP obtained before (●) and after (○) a bilateral nephrectomy in a representative sheep intravenously 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.

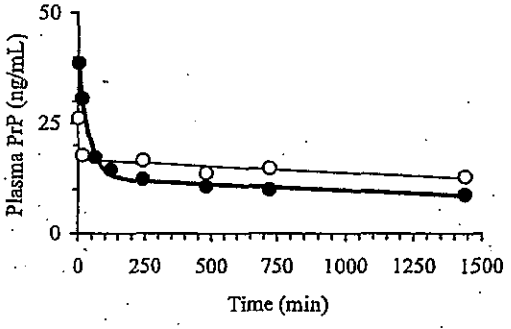

Fig. 6. Plasma PrPres concentrations after administration of SAF solution. The symbols represent the observed plasma concentrations of PrPres obtained with the PIgn-coated beads extraction method (○) or after a 60°C heat treatment (●) in a sheep intravenously administered an SAF solution containing a 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.

ACKNOWLEDGMENT

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