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Further studies of blood infectivity in an experimental model of transmissible spongiform encephalopathy, with an explanation of why blood components do not transmit Creutzfeldt-Jakob disease in humans

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BACKGROUND: Solid evidence from experimentally infected animals and fragmentary evidence from naturally infected humans indicate that blood may contain low levels of the infectious agent of Creutzfeldt-Jakob disease (CJD), yet blood components have never been identified as a cause of CJD in humans.

STUDY DESIGN AND METHODS: Blood components and plasma fractions were prepared from the pooled blood of mice that had earlier been infected with a mouse-adapted strain of human transmissible spongiform encephalopathy (TSE). Infectivity bioassays were conducted in healthy mice, and the brains of all assay animals dying during the course of the experiments were examined for the presence of proteinase-resistant protein.

RESULTS: Infectivity in the blood during the preclinical phase of disease occurred in the buffy coat at infectious unit (IU) levels between 6 and 12 per mL and was either absent or present in only trace amounts in plasma and plasma fractions. Infectivity rose sharply at the onset of clinical signs to levels of approximately 100 IU per mL of buffy coat, 20 IU per mL of plasma, 2 IU per mL of cryoprecipitate, and less than 1 IU per mL of fractions IV and V. Plasma infectivity was not eliminated by either white cell-reduction filtration or high-speed centrifugation. Approximately seven times more plasma and five times more buffy coat were needed to transmit disease by the intravenous route than by the intracerebral route.

CONCLUSION: Epidemiologic evidence of the absence in humans of disease transmission from plasma components can probably be explained by 1) the absence of significant plasma infectivity until the onset of symptomatic disease, and comparatively low levels of infectivity during the symptomatic stage of disease; 2) the reduction of infectivity during plasma processing; and 3) the need for at least five to seven times more infectious agent to transmit disease by the intravenous than intracerebral route. These and other factors probably also account for the absence of transmission after the administration of whole blood or blood components.

In previous studies of normal human blood "spiked" with scrapie-infected brain cells and of blood from mice inoculated with a human strain of transmissible spongiform encephalopathy (TSE), we found progressively lower levels of infectivity in buffy coat, plasma, and plasma Cohn fractions.[1] The present study extends these observations in new experiments using the TSE mouse model, looking particularly at infectivity during the preclinical phase of disease, at the influence of processing steps on residual plasma infectivity, and at the relative efficiency of disease transmission by the intravenous rather than intracerebral route of inoculation. The assembled data provide a plausible explanation for the fact that, despite the possible presence of the infective agent in the blood of some donors who later die of Creutzfeldt-Jakob disease (CJD), no case of transmission in humans through blood or from blood donors has ever been identified.

ABBREVIATIONS: CJD = Creutzfeldt-Jakob disease; IU(s) = infectious unit(s); PPP = platelet-poor plasma; RBC(s) = red cell(s); TSE = transmissible spongiform encephalopathy; WBC(s) = white cell(s).

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MATERIALS AND METHODS

Experimental model

Weanling Swiss Webster mice were inoculated intracerebrally with 0.03 mL of a 10-percent saline suspension of brain homogenate from mice infected with the Fukuoka-1 mouse-adapted strain of human Gerstmann-Sträussler-Scheinker disease.[2] When mice began developing symptoms about 4 months later, they were exsanguinated and the blood was pooled before separation into components and plasma fractions. Different experiments were conducted on blood collected in pools from two different groups of inoculated mice: one group of 95 mice had been inoculated in our previous experiment, and the pooled plasma from symptomatic mice (16 weeks after inoculation) had been stored at –70°C until it was used in this study. The second group of 310 mice was newly inoculated with pooled brain homogenate from mice dying during the course of the first experiment: groups of 30 mice were exsanguinated when still asymptomatic at 5 weeks, 9 weeks, and 13 weeks after inoculation, and the remaining 220 mice were exsanguinated at an early stage of illness, 18 weeks after inoculation.

Separation of blood into components and plasma fractions

Our protocols for separation of whole blood into red cell (RBC), buffy coat, and plasma components and for the further processing of plasma into Cohn fractions were described in detail in a previous publication.[1] Briefly, blood was centrifuged for 4 minutes at 2280 × *g*, and the platelet-rich supernatant plasma was removed from the RBC-buffy coat sediment (which was frozen intact at –70°C) and recentrifuged at 4200 × *g* for 8 minutes. The supernatant platelet-poor plasma (PPP) was fractionated successively into cryoprecipitate, fraction I+II+III, fraction IV, and fraction V, by a series of cold precipitations carried out in a refrigerated circulating bath, using different ethanol concentrations and pH conditions in a modified Cohn procedure.[3] Yields of the fraction pastes (mg/mL plasma) were comparable to those obtained in commercially prepared fractions from human plasma. A modification of the Cohn-Oncley cold ethanol precipitation procedure was used for separating fraction I+III from fraction II in the combined fraction I+II+III.[4] Before inoculations were begun, the frozen surface layer of buffy coat was cut away from the underlying RBC sediment and then thawed.

Filtration and centrifugation experiments

For experiments on white cell (WBC)-reduction filtration, PPP was filtered after a single freeze-thaw cycle or was filtered fresh without having been frozen and thawed. The plasma was very gently passed through 28-mm diameter filters (PLF1, Pall Corp., Port Washington, NY) at a rate of about 1 drop per second. Validation of WBC reduction efficiency was established by using buffy coat-enriched normal mouse plasma, which reduced WBCs from 7.5×10^7 per mL to below the threshold of detection (3×10^3/mL) in the filtrate. For the high-speed centrifugation experiment, 2 mL of PPP was centrifuged for 30 minutes at 17,000 × *g*, after which the supernatant was decanted, and the nearly invisible pellet was resuspended in 0.4 mL of saline.

Evaluation of residual cells and particulate matter in centrifuged plasma

To assess the presence of residual WBCs in PPP, we examined 3 units of fresh human source plasma and 3-unit equivalents of recovered plasma that had been separated by blood banks using the same centrifugation times and speeds used in our mouse blood protocol. Aliquots of each plasma specimen were centrifuged for 30 minutes at 4200 × *g*, and the resuspended cell pellets were examined by light microscopy.

In addition, a 1-mL aliquot of pooled infectious mouse plasma was used to assess the presence of particulate matter in both the PPP and its supernatant after the 17,000 × *g* centrifugation. PPP was evaluated by examination of the pellet after the 17,000 × *g* centrifugation, and the supernatant from this centrifugation was in turn evaluated by examination of the pellet after centrifugation for 2 hours at 436,000 × *g* (sufficient to band free hemoglobin in the lower quarter of the centrifuge tube). Both pellets were embedded in methacrylate and examined by transmission electron microscopy.

Infectivity assays

Weanling Swiss Webster mice were inoculated intracerebrally with 0.03 mL of specimens that were either undiluted or in dilutions ranging from 1-in-2 through 1-in-40, depending on the specimen being assayed. Some mice received 0.15 to 0.2 mL of either undiluted plasma or 1-in-4 diluted buffy coat administered through the tail vein. The observation period of the assay mice was terminated 14 months after inoculation. Fifty-five uninoculated sentinel mice remained alive and healthy throughout the observation period.

Proteinase K-resistant protein immunoassays

The brains of all mice dying during the course of the observation period and brains from 60 randomly selected mice that were still asymptomatic at the termination of the experiment were removed and frozen at –70°C. Freshly thawed brain tissue was homogenized in 9 volumes of lysis buffer (100 m*M* sodium chlorate, 10 m*M* EDTA, 0.5% NP40, 0.5% sodium deoxycholate, 10 m*M* Tris, pH 7.4) and aliquots were digested with 100 µg per mL of proteinase K and processed as previously described.[5] Samples were resolved on 16-percent polyacrylamide gels (Tris-Glycine gels, NOVEX, San Diego, CA) and electroblotted to nitro-

cellulose membranes (NEN Life Science Products, Boston, MA). Proteinase K-resistant protein (PrP) was detected by using a 1-in-100,000 dilution of a mouse monoclonal antibody[6] (6H4, Prionics, Zürich, Switzerland). The blots were developed using a Western blot chemiluminescence reagent for horseradish peroxidase detection (Renaissance, NEN Life Science Products). Signals were recorded on film (X-OmatBlue XB-1, Eastman Kodak Company, Rochester, NY).

This protocol usually produced clearly negative or positive staining results, but, in a small number of cases where staining was equivocal, fresh aliquots of brain were similarly extracted and then concentrated before electrophoresis, and duplicate aliquots were extracted by using a modification of Diringer's purification method, after which PrP was detected by using 3F4 mouse monoclonal antibody.[7] Both methods gave unequivocal and corroborative positive or negative results for all retested specimens. Brain extracts from all of the 60 asymptomatic inoculated mice that were euthanized at the conclusion of the observation period tested negative for PrP.

Infectivity calculations

Because the morphologic identity of the infectious particle in TSE is unknown, infectivity must be defined functionally; that is, the minimum amount of infectious material that is capable of transmitting disease is considered to contain a single infectious unit (IU). This functionally defined quantity depends on the route of inoculation used to bioassay the infectious material. For optimal detection, the intracerebral route of inoculation is used, and, unless otherwise indicated, all reference to infectivity in this study implies the intracerebral inoculation of assay specimens.

Because IUs cannot be observed directly, estimates of infectivity levels and statistical comparisons of these levels can be obtained only in the context of a probabilistic model for disease transmission. The probabilistic model assumed for the statistical estimation and testing methods employed here is the one-hit model proposed by Peto,[8] which has previously been used for analysis of virus assays.[9] It is assumed that IUs occur in the inoculated specimen according to a Poisson distribution, test animals are homogeneous, and a single (functionally defined) IU will transmit disease. Maximum-likelihood methods are used to estimate infectivity levels and make statistical comparisons between them. Methodologic details are provided in Appendix A.

Calculations of transmission risk

Estimates of the risk of disease transmission from contaminated plasma pools are based on the use of a binomial distribution analysis. The probabilities that a CJD donor would contribute to a plasma pool and that a therapeutic dose of plasma protein concentrate would contain sufficient infectivity to transmit disease depend on a number of factors. These factors include the prevalence of infectious CJD cases in the donor population, the size of the donor pools used to produce plasma components, the plasma volume required to process one vial of protein concentrate, the number of IUs contained in an infected donation, and the minimum number of IUs required to transmit disease when the units are administered by different inoculation routes.

In particular, the disparity between the amounts of infectious material required to transmit disease by intracerebral or intravenous routes (described in Results) can be interpreted in ways that yield markedly different transmission risk estimates. One interpretation is that more IUs are required to produce an infection by intravenous inoculation than by intracerebral inoculation. An alternative interpretation is that, even with intravenous administration, it takes only one IU to transmit disease, but only a portion of the IUs given intravenously will reach the brain and transmit disease. Details of the risk calculation methods for each interpretation are provided in Appendix B.

RESULTS

Infectivity during the preclinical and clinical stages of disease and the limits of infectivity detection

Table 1 compares levels of infectivity found in the blood of symptomatic mice in the present study to those found in our earlier study[1]; although absolute levels were higher in the present study, the hierarchy of greater and lesser levels was comparable in both studies. The much higher level of infectivity in buffy coat was not unexpected, as we noted in our earlier report that some mice receiving buffy coat had died with typical clinical signs, but they were not included in our calculations because their brains were not examined

TABLE 1. Infectivity levels in blood components and Cohn plasma fractions of symptomatic mice infected with the Fukuoka-1 strain of TSE: comparison to infectivity levels from our previous experiment*

	Number of IUs/mL		
	Present experiments		Previous
	Pool 1	Pool 2	experiment
Brain	NT	10^7	10^6
Buffy coat	NT	106.0	44.4
Plasma	34.4	21.9	10.3
Cryoprecipitate	2.6	NT	1.2
Fraction			
I+II+III	NT	NT	0.8
IV	0.5	NT	0.0
V	0.0	0.3	0.0
V supernatant	0.0	NT	0.0

* Details of the method for calculating infectivity levels shown in this and all subsequent tables are described in Appendix A. Values from the previous experiment were recalculated by using the same Poisson distribution analysis as in the present experiment.

TABLE 2. Infectivity levels in the buffy coat, plasma, and Cohn cryoprecipitate + fraction I+II+III in groups of mice exsanguinated at intervals of 5, 9, 13, and 18 weeks after inoculation with the Fukuoka-1 strain of TSE

Time after inoculation (weeks)	Specimen*	Inoculated volume (mL)†	Number of inoculated animals	Number of positive animals	IUs per mL (95% CI)
5	Buffy coat	0.28	35	3	11.3 (3-29)
	Plasma	0.59	40	1	1.8 (0.1-8)
	Cryo+I+II+III	0.16	29	2	0.5 (0.1-1.5)
9	Buffy coat	0.15	29	1	6.8 (0.4-30)
	Plasma	0.29	38	0	0.0 (0-11)
	Cryo+I+II+III	0.14	28	3	1.0 (0.3-2.6)
13	Buffy coat	0.16	28	1	6.4 (0.4-28)
	Plasma	0.29	38	0	0.0 (0-11)
	Cryo+I+II+III	0.15	28	2	0.6 (0.1-1.7)
18‡	Buffy coat	0.14	27	11	106.0 (55-184)
	Plasma	0.25	33	5	21.9 (8-47)

* Cryoprecipitate and fraction I+II+III (Cryo+I+II+III) were prepared from 10 mL of plasma.
† 0.03-mL volumes were inoculated into groups of mice either undiluted or at dilutions of 1-in-2, 1-in-4, or 1-in-40. These dilution factors were taken into account in calculations of the total undiluted inoculated volume of each specimen.
‡ Cryoprecipitate from this bleeding was not available for inoculation.

for the presence of PrP, and so death was not proven to have resulted from disease transmission. Fractions IV and V, in which infectivity was not previously detected, caused one (Fraction V) or two (Fraction IV) transmissions in the present study.

Table 2 shows the levels of infectivity in buffy coat, plasma, and a combined cryoprecipitate and fraction I+II+III preparation at 5 weeks, 9 weeks, and 13 weeks after inoculation and in buffy coat and plasma at the onset of illness (18 weeks after inoculation). It is evident that preclinical plasma infectivity levels (like those of fractions IV and V prepared from plasma of symptomatic mice) were at the limit of assay recognition (sometimes below and sometimes above) and were detectable only by the inoculation of larger numbers of animals than are usually used for infectivity assays. Even if the levels of infectivity in the 5-week buffy coat and plasma specimens represent host-replicated agent, and not merely residual infectivity from the inocula, they were not significantly different from the lower (buffy coat) or absent (plasma) levels found in the 9- and 13-week specimens ($p>0.35$). In contrast, all of these incubation-period infectivity levels were significantly lower than the corresponding 18-week specimens from symptomatic animals ($p<0.006$), and, at 18 weeks, the infectivity of plasma was significantly lower than that of buffy coat ($p<0.003$).

Effect of plasma fraction processing

When fraction I+II+III was subjected to a selective precipitation of fraction I+III, leaving fraction II (the source of IgG) in the supernatant fluid, most of the infectivity followed the precipitate ($p<0.001$) (Table 3). A similar effect of precipitate removal of infectivity was evidenced by the lower levels of infectivity in successive Cohn fractions, observed in both our present study and previous studies.[1]

TABLE 3. Infectivity in subfractions of Cohn fraction I+II+III from mice inoculated with the Fukuoka-1 strain of TSE

Specimen	Inoculated volume (mL)*	Number of inoculated animals	Number of positive animals	IUs per mL plasma (95% CI)
I+III	0.54	18	17	6.2 (3.3-12)
II	0.48	16	2	0.3 (0.04-0.83)

* 0.03-mL volumes were inoculated intracerebrally at dilutions of 1-in-4 (fraction I+III) or 1-in-20 (fraction II). These dilution factors were taken into account in calculations of the total undiluted inoculated volume of each specimen.

TABLE 4. WBC-reduction filtration of plasma from mice inoculated with the Fukuoka-1 strain of TSE

Experimental conditions	Specimen	Inoculated volume (mL)*	Number of inoculated animals	Number of positive animals	IUs per mL (CI)[2]
Experiment 1:					
16-week plasma pool (symptomatic mice)					
Frozen and thawed plasma	Prefiltration	0.47	28	12	34.4 (18-58)
(undiluted)	Postfiltration	0.33	35	4	13.0 (4-30)
Experiment 2:					
13-week plasma pool (asymptomatic mice)					
Frozen and thawed plasma	Prefiltration	0.29	38	0	0.0 (0-11)
(diluted 1-in-4)	Postfiltration	0.26	35	8	34.6 (16-65)
Experiment 3:					
18-week plasma pool (symptomatic mice)					
Fresh plasma	Prefiltration	0.25	33	5	21.9 (8-47)
(undiluted)	Postfiltration	0.23	29	4	19.8 (6-46)

* 0.03-mL volumes were inoculated intracerebrally at dilutions of 1-in-2 and 1-in-4. These dilution factors were taken into account in calculations of the total undiluted inoculated volume of each specimen.

Effect of WBC-reduction filtration on plasma infectivity

Three experiments were conducted to assess the ability of WBC-reduction filtration to reduce or eliminate infectivity present in PPP (Table 4). In the first two experiments (using frozen and thawed plasma), infectivity was either marginally reduced ($p = 0.07$) or significantly increased ($p<0.001$). In the third experiment (using fresh plasma), infectivity levels observed in prefiltration and postfiltration specimens were nearly identical ($p = 0.88$).

Effect of centrifugation on plasma infectivity

PPP from units of either recovered or source plasma from normal human blood donors contained between 10 and 200 intact WBCs per mL (average, 80 cells/mL). In a further attempt to eliminate WBCs (and infectivity) from infectious mouse plasma, the PPP was subjected to high-speed centrifugation, and an aliquot of the supernatant plasma was assayed in parallel with a saline suspension of the barely visible pellet. As shown in Table 5, plasma infectivity was only marginally reduced by this procedure (plasma vs. supernatant, $p = 0.01$; pellet vs. supernatant, $p = 0.07$). Electron microscopic examination of the pellet from PPP following centrifugation at 17,000 × *g* revealed numerous cell ghosts and cytoplasmic granules. Examination of the pellet from that supernatant following further centrifugation at 436,000 × *g* revealed only occasional electron-dense spheres of undetermined origin, with a diameter of approximately 10 nm and no delimiting membranous structure.

Comparative efficiency of intravenous and intracerebral routes of inoculation in transmitting disease

Two experiments were conducted: buffy coat from one mouse pool and PPP from the other mouse pool were used in parallel intracerebral and intravenous assays (Table 6). Approximately five times more buffy coat and seven times more plasma were required to transmit disease by the intravenous route than by the intracerebral route ($p<0.001$).

DISCUSSION

Of all the results obtained from this study, it seems to us that two observations stand out as particularly important: the absence of significant amounts of infectivity in the plasma of animals in the preclinical phase of TSE, and the requirement for the inoculation of five to seven times more infective material to transmit disease by the intravenous route than by the intracerebral route.

Published estimates of the probability that a donor later dying of CJD would contaminate plasma pools of varying sizes have been based on the prevalence of CJD in the donor population and on informed guesses about the length of time that blood might be infectious before the onset of symptoms and (with data from rodent experiments) the level of infectivity that might be expected to occur.[10,11] The surprising conclusion was that the risk of infection from a vial dose of protein concentrate would be such that 1 out of every 7 to 10 recipients should come down with CJD. Clearly, something was wrong with the set of assumptions leading to this conclusion, as no case of iatrogenic CJD in a blood component or product recipient has ever been identified. Our new experimental data provide at least a partial explanation for this anomaly.

Preclinical versus clinical disease; intracerebral versus parenteral infection

If, as in experimentally infected mice, plasma from humans with CJD contains little or no infectivity during the preclinical phase of disease, and if disease transmission through intravenous administration requires five to seven times more infectivity than that through intracerebral inoculation (a figure similar to the 9:1 intravenous-intracerebral ratio previously observed in mice inoculated with scrapie brain

TABLE 5. Centrifugation for 30 minutes at 17,000 × *g* of plasma from mice inoculated with the Fukuoka-1 strain of TSE

Specimen	Inoculated volume (mL)*	Number of inoculated animals	Number of positive animals	IUs per mL (95% CI)
Plasma	0.47	28	12	34.4 (18-58)
Pellet†	0.26	34	19	21.8 (13-34)
Supernatant	0.30	40	2	6.8 (1.1-21)

* 0.03-mL volumes were inoculated intracerebrally at a dilution of 1-in-4. This dilution factor was taken into account in calculations of the total undiluted inoculated volume of each specimen.
† Resuspended in saline.

TABLE 6. Bioassay of infectivity in the plasma and buffy coat of symptomatic mice inoculated 16 to 18 weeks earlier with the Fukuoka-1 strain of TSE: comparison of levels of infectivity detected by intracerebral or intravenous inoculation of specimens into the assay animals

Specimen	Route of inoculation	Inoculated volume*	Number of inoculated animals	Number of positive animals	IUs per mL (95%CI)[2]	Intravenous to intracerebral equivalence
Plasma	Intravenous	4.50	30	16	5.1 (3-8)	
	Intracerebral	0.47	28	12	34.4 (18-58)	7:1
Buffy coat	Intravenous	1.40	28	18	20.6 (12-32)	
	Intracerebral	0.14	27	11	106.0 (55-184)	5:1

* For intracerebral inoculations, 0.03 mL was inoculated at dilutions of 1-in-2, 1-in-4, or 1-in-40. For intravenous inoculations, 0.15 mL was inoculated undiluted (plasma) or 0.2 mL at a dilution of 1-in-4 (buffy coat). These dilution factors were taken into account in calculations of the total undiluted inoculated volume of each specimen.

homogenate[12]), new sets of risk calculations can be formulated. We can now distinguish two quite different situations: one in which blood donations are made by individuals who only years later develop clinical signs, and another in which blood donations are made by individuals in the immediately preclinical or early clinical phase of CJD. The first (common) situation will involve a comparatively long duration but low level of plasma infectivity, and the second (rare) situation a much shorter duration but higher level of plasma infectivity.

If we interpret the much greater amount of plasma needed to transmit disease intravenously (7×) than intracerebrally as indicating that 7 IUs are needed to transmit disease by the intravenous route, the calculated risk of transmitting disease from a pool containing a donation from an individual in the preclinical stage of CJD varies from approximately 1 in 10 billion to less than 1 in a trillion, depending on the size of the donor pool (Table 7). Halving or doubling the estimated preclinical period changes the risk estimates by about one order of magnitude in either direction. These very low risk probabilities could by themselves explain the epidemiologic absence of transmission in recipients of recovered plasma pools. However, in the immediate preclinical and clinical phase, in which infectivity levels are higher, the calculated risks are also higher, ranging from approximately 1 in 500,000 (10,000-donation pool) to 1 in 100 million (100,000-donation pool).

If we interpret the 7:1 intravenous-intracerebral ratio as indicating that, although a single IU is capable of transmitting disease, only 1 of 7 intravenously administered IUs reaches the brain, most of this "statistical protection" disappears. The probability that a vial dose of plasma could transmit disease increases to more than 1 in 1000, irrespective of the stage of disease or donor pool size (Table 8).

The calculated risks would be even higher with professional donation of source plasma, a situation in which a donor could contribute as much as 10 times more plasma (2500 mL) to a pool than would an individual whose plasma was recovered from a single donation (250 mL). In this situation, the probability that a vial dose from a random pool would be able to transmit CJD could be as high as 3 to 4 percent, which, like earlier estimates of risk, is incompatible with the observed absence of CJD in epidemiologic studies of plasma recipients.

Reduction of infectivity by plasma processing

If we cannot rely on probabilities alone to explain the apparent lack of risk in plasma recipients, we still have to take into account the reductions in infectivity associated with plasma fractionation and processing into therapeutic protein concentrates. We have shown that crude plasma fractions contain only one-tenth (or less) the amount of infectivity present in unfractionated plasma and that an additional precipitation of fraction I+III away from fraction II results in a further log loss of infectivity. A mass of emerg-

TABLE 7. Probability that a vial of plasma protein concentrate made from a donor pool to which an individual with CJD had contributed would contain a transmissible amount of infectivity, if 7 intravenously administered IUs are needed to transmit disease

Donor pool size	Probability that pool will include at least one CJD-infectious donor (%)	Probability that a vial dose from a pool with one CJD-infectious donor will be capable of transmitting infection*	Probability that a vial dose from a random pool will be capable of transmitting infection*
Preclinical phase of CJD (10 years, 1 IU/mL plasma)			
10,000	12.7	7.8×10^{-11}	1.5×10^{-10}
50,000	49.3	1.1×10^{-15}	2.4×10^{-13}
100,000	74.3	$<10^{-16}$	2.5×10^{-14}
Clinical phase of CJD (1 year, 20 IUs/mL plasma)			
10,000	1.4	1.4×10^{-2}	2.1×10^{-4}
50,000	6.6	1.0×10^{-6}	2.9×10^{-7}
100,000	12.7	9.7×10^{-9}	1.5×10^{-8}

* Assumes that a vial of protein concentrate is processed from the plasma volume contained in 5 blood donations.

TABLE 8. Probability that a vial of plasma protein concentrate made from a donor pool to which an individual with CJD had contributed would contain a transmissible amount of infectivity, if 1 IU is sufficient to transmit disease, but only 1 of 7 intravenously administered IUs reaches the brain

Donor pool size	Probability that pool will include at least one CJD-infectious donor (%)	Probability that a vial dose from a pool with one CJD-infectious donor will be capable of transmitting infection*	Probability that a vial dose from a random pool will be capable of transmitting infection*
Preclinical phase of CJD (10 years, 1 IU/mL plasma)			
10,000	12.7	1.8×10^{-2}	2.4×10^{-3}
50,000	49.3	3.6×10^{-3}	2.4×10^{-3}
100,000	74.3	1.8×10^{-3}	2.4×10^{-3}
Clinical phase of CJD (1 year, 20 IUs/mL plasma)			
10,000	1.4	3.0×10^{-1}	4.1×10^{-3}
50,000	6.6	6.9×10^{-2}	4.7×10^{-3}
100,000	12.7	3.5×10^{-2}	4.8×10^{-3}

* Assumes that a vial of protein concentrate is processed from the plasma volume contained in 5 blood donations.

ing experimental data from other laboratories shows that virtually every processing step involving precipitation, filtration, or chromatography is associated with removals of between 1 and 6 log of infectivity, with an average reduction of 3 to 4 log (Rohwer R, Drohan W; Pettaway S; and Bailey A, unpublished, independent data). These removals would reduce the statistical risk of disease transmission to even lower levels than were estimated for unprocessed plasma from preclinical donors.

The nontransmissibility of human CJD by whole blood or its cellular components probably also results from a combination of factors. In whole-blood transfusion experiments involving 3 terminally ill donor mice and 20 recipient mice, we observed transmission in only a single animal, and similar experiments in scrapie-infected hamsters have been even less successful (Rohwer R, Drohan W: unpublished data). Moreover, whole-blood transfusions in humans have markedly declined in recent years in favor of the use of "targeted" blood components or non-blood-derived fluids, and packed RBCs and platelets probably contain little or no infectivity (our study did not examine this point, but preliminary results in a scrapie-infected hamster model indicate that both of those components have lower levels than either buffy coat or plasma (Rohwer R, Drohan W, unpublished data).

Buffy coat would pose the greatest potential risk of disease transmission, because it has the highest infectivity concentration of any blood component, but mononuclear cells are not used therapeutically at the present time and granulocyte transfusions are uncommon. Moreover, human granulocytes do not produce PrP, and thus are unlikely to be capable of transmitting infection.[13] WBCs are a source of human interferon, but the only Food and Drug Administration-approved products undergo at least two chromatography steps during processing, with predicted large losses of any infectivity that might have been present in the starting material.

Rodent versus human infection

None of these considerations can satisfactorily explain the apparent inability of unprocessed plasma (especially source plasma) to transmit disease, which brings us, finally, to a consideration of similarities and differences between experimentally induced disease in rodents and naturally occurring disease in humans. Similarities include the pathogenetic progression of disease after peripheral infection and the uniform finding of higher infectivity titers in central nervous system tissue than in peripheral tissue. Differences include the fact that absolute levels of infectivity in any given tissue can vary widely between natural and experimental disease, as is illustrated by the fact that infectivity in brains from patients dying of CJD averages about 40,000 IU per g of tissue,[14] whereas infectivity levels were 25 to 250 times higher in the two brain pools from our mice, and the higher of the two pool titers was associated with the higher blood infectivity levels.

Furthermore, blood from naturally infected animals (sheep with scrapie and cattle with bovine spongiform encephalopathy) is not infectious, and the detection of infectivity in blood from humans with CJD has been irregular, with each of the few successes—obtained by the intracerebral inoculation of rodents—open to question on technical grounds.[15] These isolations must also be judged in light of numerous failures to detect infectivity by using the two most sensitive bioassay systems: nonhuman primates[14] and "humanized" transgenic mice (Prusiner S, unpublished data, June 1999).

For all of these reasons, we believe that our rodent model almost surely overestimates the levels of infectivity present in the blood of humans with naturally occurring CJD and that, although unquantified, the actual risk of such blood to contain sufficient infectivity to transmit disease when administered intravenously to another human must be very close to zero.

Donor pool size and apparent dilution-related reduction in transmission risk

We have previously argued that an IU, which by definition is capable of transmitting infection, should retain that capability whether contained in the plasma from a pool of 10 or 10 million donations; that is, it will never be "diluted-out."[11] This reasoning assumed that infectivity would be transmitted in the same way it was assayed—that is, by intracerebral inoculation; however, the need for more infectivity to transmit disease when the inoculation is given by the intravenous rather than the intracerebral route significantly affects the calculation.

At some dilution, the amount of infectivity contained in the volume inoculated into a single animal, although insufficient to transmit disease by the intravenous route, should still be capable of transmitting disease by the intracerebral route. This should occur whether parenteral transmission requires multiple IUs, or merely enough "excess" IUs to surmount peripheral clearance mechanisms. The predicted disappearance of infectivity in specimens inoculated intravenously is not inconsistent with the argument that an IU will remain infectious no matter how great the dilution. It makes no assumptions about the physical state of the IU; it only says that more infectivity is required to transmit disease by the intravenous route than by the intracerebral route. Assuming that levels of human plasma infectivity are as low as or lower than those in mice, a practical consequence is that larger plasma pools would be predicted to reduce the risk of CJD transmission.

WBC-reduction filtration and high-speed centrifugation

The failure of filtration or high-speed centrifugation to eliminate plasma infectivity has both practical and theoreti-

cal implications. The much higher levels of infectivity in buffy coat than in plasma in endogenously infected pools of blood, as well as the similar distribution of infectivity in normal human blood that had been "spiked" with intact infectious brain cells, strongly suggests that the origin of blood infectivity is to be found in the WBC. Although WBC-reduction filtration of whole blood to reduce the number of WBCs admixed in packed RBCs is a plausible strategy to decontaminate the RBCs, WBC-reduction filtration of plasma to eliminate residual infectivity does not appear to be useful.

The failure of WBC-reduction to reduce infectivity indicates that intact cells are not the source of plasma infectivity. The failure of high-speed centrifugation to eliminate infectivity in plasma that contained no detectable membranous structures also indicates that fragmented membranes from WBCs and/or platelets in the plasma are not the only source of infectivity. Therefore, if plasma infectivity originates in WBCs, it must be partly in the form of very small, unsedimentable particles, molecular aggregates, or individual molecules in the cytoplasm of disrupted cells, or released from their membranes. Such nonsedimentable, nonmembrane-associated particles that have not been subjected to artefact-inducing extraction procedures could prove useful in experimental efforts to measure the size and explore the nature of the smallest naturally occurring molecular species capable of transmitting disease.

We believe that the experiments reported here and in our previous article[1] have culled about as much information from the TSE-infected mouse model as can be usefully applied to the human situation. It will be important to learn if other experimental models, especially CJD-infected nonhuman primates, yield data that support the conclusions drawn from the mouse model and validate our explanation of why, despite the possibility that some blood donor pools may indeed be "tainted" with infectivity, no sporadic, familial, or iatrogenic CJD has ever been transmitted to recipients of blood or blood components or products. Additional experiments, already underway, will be needed to assess the risk, if any, from the blood of patients with new variant CJD.

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APPENDIX A. MAXIMUM LIKELIHOOD ESTIMATION AND TESTING OF INFECTIVITY LEVELS

The likelihood functions for the data in our experiments are modeled in the same form as the one-hit model of Peto.[8] It

is assumed that IUs occur in a specimen of volume v, according to a Poisson distribution with rate parameter k, where k denotes the functional infectivity level. By "functional," it is meant, for example, that if IUs occur at a rate of 2 per unit volume, but only 50 percent will be capable of transmitting disease under the conditions of specimen processing and inoculation, then the functional infectivity level is 50 percent of 2, or 1 IU per unit volume. Alternatively, if groups of 2 IUs are required to transmit disease, then the functional infectivity level is still 1 IU per unit volume.

The probability that an inoculated animal will become infected is equal to the probability that it receives at least 1 IU capable of transmitting disease, which under the Poisson model is $1 - \exp(-kv)$, where exp is the inverse natural logarithm, k is the functional infectivity level, and v is the inoculated volume. Then, the likelihood function (L) for the data observed on n mice is given by:

$$L(k) = (1 - \exp(-kv_1))^{y_1} \times (1 - \exp(-kv_2))^{y_2} \times \ldots \times (1 - \exp(-kv_n))^{y_n} \times (\exp(-kv_1))^{1-y_1} \times (\exp(-kv_2))^{1-y_2} \times \ldots \times (\exp(-kv_n))^{1-y_n}$$

where v_i is the volume of undiluted specimen inoculated into the ith mouse, and y_i is an indicator of infection equal to 1 if the ith mouse becomes infected, or equal to zero otherwise.

Standard maximum likelihood estimation and testing methods are then applied. (See, for example, McCullagh P, Nelder JA. Generalized linear models. 2nd ed. New York, Chapman & Hall, 1989:469-73.) The maximum likelihood estimation of k is the value k^* that maximizes the function L(k). Likelihood ratio-based 95% CIs are obtained by finding the two roots of the equation

$$2[\log(L(k^*)) - \log(L(k))] = 3.84.$$

When all y_i are identically zero (i.e., no animals become infected), an upper 95% CI for k can be calculated as the solution to the equation

$$\exp(-kv_1) \times \exp(-kv_2) \times \ldots \times \exp(-kv_n) = 0.05.$$

A test for comparing infectivity levels k_1 and k_2, from two different experiments, is based on the likelihood ratio statistic

$$2[\log(L_1(k_1^*)) + \log(L_2(k_2^*))] - 2\log(L(k^*))$$

where $L_1(k_1^*)$ and $L_2(k_2^*)$ are the maximized likelihoods from the two experiments individually, and $L(k^*)$ is the maximized likelihood computed from the data pooled from the two experiments.

Tests are based on the fact that this statistic has an approximate chi-square distribution with one degree of freedom. For cases in which there is a negligible chance that more than 1 IU could be present in the volume inoculated into any one animal (for example, when the infectivity level is very low and the inoculated volumes are very small), it can be shown mathematically that a simple, approximate estimate of functional infectivity level is available. That estimate is calculated by dividing the total number of animals that become infected by the total volume of undiluted specimen inoculated into the group of test animals.

APPENDIX B. RISK CALCULATION METHODS

To compute the prevalence of infectious donors, it is assumed that, in the general population, the incidence of CJD is approximately 1 case per million people per year and that individuals who are incubating CJD donate blood at about the same rate as the general population. If there is a 10-year period during which the blood of an individual incubating CJD is potentially infective, then the prevalence of infective cases is 10 per million. The donor population is mainly in the 20- to 75-year-old age range, and this group represents approximately 65 percent of the total US population and accounts for 89 percent of all cases of CJD. The approximate prevalence of CJD-infective individuals, age-adjusted to the donor population, is (0.89/0.65) × 10 per million, or 13.7 per million donors (this approximation ignores the fact that an individual may transition into or out of the donor age range during the period of infectivity). If a 1-year period of infectivity is assumed, then the approximate prevalence of CJD-infective individuals in the donor population is 1.37 per million.

The probability that a plasma pool will include at least one CJD-infectious donor increases with the prevalence of CJD-infectious donors in the donor pool and with the size of the pool. Assuming that individuals in the donor population contribute to pools at random, the probability that (d) CJD-infectious donors are included in a pool of (N) donors is given by the binomial probability:

$$p_d = \{N!/(N - d)!d!\}(\text{prevalence})^d(1 - \text{prevalence})^{N-d}$$

where $N! = 1 \times 2 \times 3 \times \ldots \times N$, and d! is defined similarly. It follows that the probability that a pool of (N) donors contains at least one CJD-infectious donor is $1 - (1 - \text{prevalence})^N$.

The probability that a single vial processed from a pool including a single donation from an individual with CJD will be infectious depends on the total number of IUs in a single infected donation (I), the minimum number of independent IUs (i) required to transmit disease when given intravenously, and the fraction (f) of the pool represented by the plasma volume required to process one vial of protein concentrate. This probability can be calculated as the sum of binomial probabilities:

$$r(i,I,f) = \{I!/[(I - i)!i!]\}f^i(1 - f)^{I-i} + \{I!/[(I - i - 1)! \times (i + 1)!]\}f^{i+1}(1 - f)^{I-i-1} + \{I!/[(I - i - 2)!(i + 2)!]\}f^{i+2}(1 - f)^{I-i-2} + \ldots + f^I.$$

Moreover, considering the possibility that any number of CJD-infectious donors will contribute to the pool, the overall probability that a single vial processed from any pool will be infectious is the weighted average of risks of an infectious vial, with weights being the probabilities of various

numbers of infectious donors being included in the pool. This overall probability is

$$R(i,I,f) = p_1 \times r(i,I,f) + p_2 \times r(i,2I,f) + p_3 \times r(i,3I,f) + \ldots + p_N \times r(i,NI,f),$$

and it is based on the approximating assumption that exactly (I) IUs are contained in the donation from any infectious donor.

Two models are possible for calculating the risk of CJD transmission by the intravenous administration of plasma: either 7 IUs are required to transmit disease, or only 1 in 7 IUs (each of which alone could transmit disease) will reach the brain. Approximate risk in either situation can be computed by using the formula for R(i,I,f) given above. Under the first model, i = 7 and I = 250 (preclinical stage) or 5000 (clinical stage), assuming that a donor contributes 250 mL of plasma. For the second model, risk is computed by using i = 1 and I = 250/7 (preclinical) or 5000/7 (clinical). Under the second model, the "effective" IUs are present in such low concentration that there is negligible chance that a single-vial dose could contain more than 1 unit. In this case, a simple approximation to the risk formula is the proportion of total pool contained in a single dose multiplied by the expected total number of "effective" IUs in the pool, which is easily shown to be f × N × prevalence x I, where I = 250/7 (preclinical) or 5000/7 (clinical).

If a therapeutic dose requires multiple vials, all derived from the same plasma pool and all administered to the patient within a short period of time, then the risk of infection associated with the collection of vials can be computed as above, except that f would now designate the fraction of the pool that the multiple vials collectively constitute. For example, if the therapeutic dose consisted of two vials administered one right after the other, then the appropriate value of f to use in the calculations above would be twice the fraction associated with a single vial. If the multiple vials are derived from different pools or are administered at separated timepoints, then computation of the infection probabilities becomes substantially more complicated than that presented here. In the latter case, assumptions would have to be made regarding the length of time IUs remain active in the body relative to the rate of administration of the vials. Because many of the commonly used plasma protein concentrates are given as a single-vial dose (e.g., factor VIII, IgG, albumin), we have chosen to analyze a case in which only a single vial is administered at any one time. ◘

Febrile and allergic transfusion reactions after the transfusion of white cell-poor platelet preparations

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BACKGROUND: Nonhemolytic transfusion reactions (NHTRs) frequently occur after platelet transfusions. White cell (WBC)-derived inflammatory cytokines can cause these reactions, but they are rarely found in WBC-poor platelet preparations. Transfusion reactions were investigated with regard to the residual WBC content in the stored platelet concentrate in two consecutive study periods.

STUDY DESIGN AND METHODS: In the first study period, platelet concentrates were WBC-reduced by bedside filtration. In the second period, all platelet concentrates were filtered before storage. Recipients who experienced transfusion reactions were examined with regard to their main clinical symptoms during and after transfusion. In the supernatant of the involved platelet concentrates, concentrations of interleukin (IL)-1β, IL-6, IL-8, tumor necrosis factor (TNF)α, macrophage inflammatory protein 1α, and RANTES were analyzed.

RESULTS: The incidence of transfusion reactions remained steady when the transfusion regimen was changed from bedside filtration to prestorage WBC filtration (1.63% and 1.56%; p = 0.84). In both periods, NHTRs were predominantly of allergic origin. Inflammatory mediators IL-1β, IL-6, IL-8, and TNFα were detectable in only a minority of platelet components involved in NHTRs. Platelet concentrates involved in allergic reactions contained high concentrations of RANTES (668 ± 223 ng/mL).

CONCLUSIONS: Prestorage WBC filtration did not reduce the incidence of these reactions, and inflammatory cytokines were of minor relevance. The proinflammatory platelet-derived chemokine RANTES, which accumulates even in WBC-reduced platelet concentrates, was associated with allergic transfusion reactions. Platelet-derived mediators may be a key to understanding NHTRs.

Nonhemolytic transfusion reactions (NHTRs) are common side effects after the transfusion of blood components.[1,2] Most of the reactions are mild, but some are life-threatening, because of severe anaphylactic shock. Whereas in the past white cell (WBC) antibodies were of foremost relevance, their impact faded with the introduction of WBC-reduction devices. Recently, contaminating inflammatory cytokines in platelet concentrates (PCs), such as interleukin (IL)-1, IL-6, IL-8, or tumor necrosis factor α (TNFα), have been considered instrumental in the origin of these reactions.[3,4] These mediators can accumulate in high concentrations in stored PCs, but their levels are very dependent on the WBC contamination.[5-7] WBC-reduced PCs contain few if any inflammatory cytokines, and prestorage filtration of PCs can circumvent the accumulation of these mediators during storage.[8-11] There is also a remarkable difference in the reported incidence of NHTRs, according to the kind of PC transfused.[12] Transfusion reactions still occur after the transfusion of WBC-reduced PCs.[1,13-16] Thus, underlying pathomechanisms besides the presence of WBC-derived inflammatory cytokines might also be involved in NHTRs.[17,18]

ABBREVIATIONS: BC = buffy coat; IL = interleukin; MIP-1α = macrophage inflammatory protein 1α; NHTR(s) = nonhemolytic transfusion reaction(s); PC(s) = platelet concentrate(s); SD = single-donor; TNFα = tumor necrosis factor α; WBC(s) = white cell(s).

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