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Differential expression of cellular prion protein on human blood and tonsil lymphocytes

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ABSTRACT

Background and Objectives. **The expression of cellular prion protein (PrPc) on the surface of peripheral lymphocytes has been previously reported, but little is known about its expression on lymphoid cells from secondary lymph organs. In this report, we compare the surface expression of PrPc on human blood lymphocytes and tonsil lymphocytes.**

Design and Methods. **This analysis was performed by cytometry on live lymphocytes isolated from healthy donors or from the tonsils of adults or children.**

Results. **Human peripheral lymphocytes and tonsillar lymphoid cells, but not erythrocytes or granulocytes, express PrPc at their surfaces. Interestingly, we found significantly less PrPc on freshly isolated tonsil lymphocytes, both B and T, than on blood cells. Although tonsil cells bear less PrPc than circulating blood lymphocytes, they are able to express high quantities of PrPc on their surface when placed in culture. However, contrary to previous results, mitogen stimulation does not affect this expression on B- or T-cells.**

Interpretation and Conclusions. **We suggest that the PrPc expression by lymphocytes may be modified by interactions occurring during intratissular migration or during cell-to-cell contacts. Whether PrPc plays a role in intracellular communication at this location, as it does in the nervous system, remains an open question.**

Key words: prion protein (PrPc), human lymphocytes, cytofluorometry

Creutzfeldt-Jakob disease (CJD), Gerstmann-Sträussler-Scheinker's syndrome (GSS), fatal familial insomnia (FFI) and Kuru in humans as well as scrapie and bovine spongiform encephalopathy (BSE) in animals are transmissible neurodegenerative diseases characterized by the accumulation of an altered form, PrPres, of a normal host molecule, PrPc.[1] PrPres is derived from PrPc through a post-translational conformational modification; PrPres is characterized by partial resistance to proteinase K digestion and insolubility in non-denaturing detergents.[2]

Very little is known about the normal function of the PrPc protein. This glycoprotein is attached via a glycosylphosphatidylinositol (GPI) anchor onto the cell membrane. Neuronal cells are the main expressors of PrPc, which may play a role in normal synaptic function, survival of Purkinje neurones[3] and maintenance of circadian rhythms and sleep patterns.[4] However, expression of PrPc is not confined to the central nervous system. PrPc mRNA has been detected in many peripheral tissues, including tissues of the reproductive tract, heart, lung and spleen[5,6] and the protein has been detected on various human cells such as keratinocytes,[7] fibroblasts,[8] and lymphoid cells.[9]

Prion proteins can be experimentally propagated by intracerebral inoculation. In such experiments, the immune status of the host has no detectable influence on propagation. Yet transmission by peripheral administration, the usual route for propagating transmissible spongiform encephalopathies, is immune-dependent.[10-12] In mouse scrapie and many experimental and natural diseases, infectious prion protein accumulation is detectable in the spleen long before the involvement of the central nervous system.[13] SCID mice, which are resistant to scrapie agent,[14] develop the disease when they are reconstituted with spleen cells.[15] However, the nature of cells supporting the initial replication of the prion is still unclear. Establishment of neurologic disease depends on prion expansion within the reticular system, which appears to trap the agent on immune-competent, non-dividing cells such as follicular dendritic cells,[16,17] support a continuous replication of the agent and permit its propagation to target nervous cells.

B-cells have also been suspected to be involved. In immune-deficient mice, mutations disrupting the differentiation and response of B-lymphocytes prevent the development of clinical scrapie.[18] However, immunodeficient mice reconstituted with bone marrow samples from PrP-deficient mice are fully susceptible to intraperitoneally inoculated prion. Because both prion replication and transport from the periphery to the central nervous system depend on cellular expression of prion protein in peripheral tissues,[19] it appears of great interest to analyze PrPc expression on mature lymphoid cells. The expression

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of PrPc on the surface of peripheral lymphocytes has already been reported, but little is known about PrPc expression on lymphoid cells from secondary lymph organs.

Design and Methods

Human peripheral blood mononuclear cells

Human peripheral blood mononuclear cells were isolated by density centrifugation on Ficoll-Paque (Pharmacia) of buffy-coat (n=12) from healthy adult donors or purified from whole blood from children. Interphase cells (lymphocytes and monocytes) were recovered, washed to eliminate contaminating residual Ficoll solution, and resuspended in the same medium. Granulocytes and erythrocytes were recovered in the pellet. The red cells were lysed with distilled water and the granulocytes were resuspended in RPMI-1640. Cells were counted and their viability was determined by the Trypan blue exclusion assay.

Isolation of tonsil lymphocytes

Tonsils, surgically removed from children (n=12) or adults (n=4) were cut into fragments, disrupted and filtered to eliminate aggregates and membrane fragments. The cell suspension obtained was rinsed three times in RPMI-1640, cells were counted and viability was tested by the Trypan blue exclusion assay.

Cell cultures and lymphoid cell activation

All lymphoid cells were grown in RPMI-1640 supplemented with 10% fetal calf serum, 2 mM L-glutamine (Merck),100 U/mL penicillin and 100 µg/mL streptomycin (Bio-Wittaker). Cultures were incubated at 37°C with 5% CO_2. Cells were activated by the addition of concanavalin A (Con A, 10 µg/mL, Boehringer, Mannheim, Germany) or phorbol myristate acetate (PMA, 10 mg/mL, Sigma) plus ionomycin (Iono, 1 µg/mL, Sigma). After 6, 15, 24 and 48 h of activation, cytofluorometry was performed to determine the PrP cell expression.

Analyses of cell surface and intracytoplasmic PrPc expression by cytofluorometry

Cytochrome or PE-conjugated mouse monoclonal antibody (Mab) recognizing human B-cells (anti-CD 19, diluted 1/25, Becton Dickinson), T-lymphocytes [(PE) anti-CD3, diluted 1/25, Becton-Dickinson] and monocytes [(PE)-anti-CD14, diluted 1/25, Becton Dickinson] were used in double indirect labeling experiments with mouse monoclonal antibodies (8G8, 3B5, diluted 1/1,000) raised against different PrP epitopes. (PE)-CD25 and (PE)-CD69 antibodies were also tested as markers of lymphoid cell activation.

Briefly, 1×10^6 cells were first incubated with anti-PrP-Mab for 30 minutes at 4°C, washed, and then incubated for 30 more minutes with fluorescein isothiocyanate-conjugated rabbit anti-mouse immunoglobulins (diluted 1/200, Prosan). Cells were washed, then incubated for 30 minutes with one of the different PE-conjugated mouse antibodies.

After washing cells were analyzed with a FACScan (Becton-Dickinson, Sunnyvale, CA, USA). Cells incubated with PE-conjugated mouse monoclonal antibody and FITC-conjugated rabbit anti-mouse immunoglobulins were used as negative controls.

In some assays, cells were permeabilized for 30 minutes at room temperature in a solution of 0.1% saponin (SIGMA) in phosphate buffer.

Results

Constitutive PrPc expression on the surface of resting human blood cells and tonsil lymphocytes

First, we analyzed by cytofluorometry surface expression of PrPc of freshly isolated blood cells from healthy donors. Lymphocytes (Figure 1a) and monocytes (Figure 1b), as detected by PrP-specific 8G8 and 3B5 mouse antibodies, demonstrated cell surface positive staining. However pure isolated granulocytes were not stained (Figure 2a-b).

Human blood lymphocytes are resting cells. Typical samples contained 63% of $CD3^+$ T-cells and 7% of $CD19^+$ B-cells. Both subpopulations were found to express the same amount of PrPc on their surfaces (Figure 3a-b). Cells incubated respectively with CD3-PE or CD19-cytochrome conjugated antibodies and the secondary FITC-conjugated rabbit anti-mouse antibody (RAM) were used as control (Figure 3c-d).

We next investigated PrPc expression on human lymphoid cells isolated from children's tonsils (n=12). The lymphoid population isolated from the tonsils contained 60% of $CD19^+$ B-cells (Figure 4a) and 29% $CD3^+$ T-cells (Figure 4b). Both subpopulations were found to express much less PrPc on their membranes than blood lymphocytes.

To ensure that PrPc expression at the lymphocyte surface was not correlated with the age of each individual, we analyzed the PrPc expression on both adults and children's lymphoid cells isolated respectively from blood and tonsil of the same patient (Figure 5a-b). In each case, lymphoid cells isolated from adult or children's tonsils were found to express less

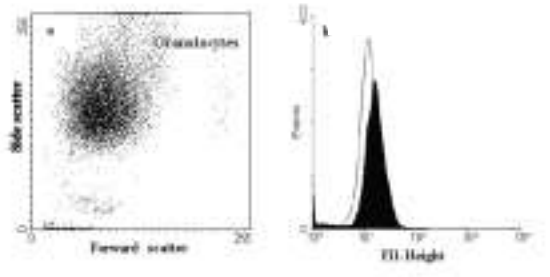

Figure 1. Expression of PrPc on human peripheral lymphocytes (a) or monocytes (b). Surface expression of PrPc on freshly isolated blood cells stained indirectly with PrP-specific 8G8 Mab (closed histograms) and rabbit anti-mouse antibody conjugated to fluorescein isothiocyanate (RAM-FITC) or secondary antibody (RAM-FITC) only (controls; open histograms). Cell number (ordinate) is plotted against fluorescence intensity (abscissa).

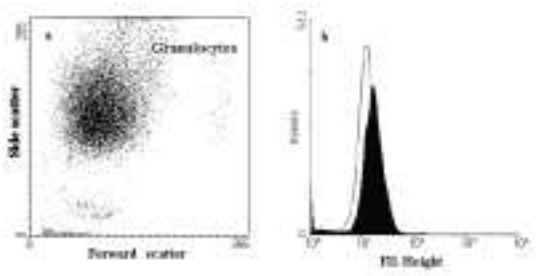

Figure 2. Cell size (forward scatter) versus cell granularity (side scatter) for freshly purified blood granulocytes (a). Granulocytes were indirectly stained with PrP-specific 8G8 Mab (b).

PrPc at their surfaces than blood cells.

In view of these results, it appeared possible that these cells may recycle their surface PrPc in cytoplasmic vesicles. We thus analyzed the potent intracytoplasmic expression of PrPc after cell membrane permeabilization. Fluorescent histograms of permeabilized cells showed that these had the same negative or low PrPc expression as did non-permeabilized tonsil lymphocytes (Figure 6a-b).

Modulation of PrPc expression on cultured tonsil lymphocytes

Tonsil lymphocytes maintained in culture conditions, without mitogen, appear to increase their PrPc expression with increasing duration of culture. Freshly isolated B- and T-tissue lymphocytes PrPc only expressed slightly at their surface (Figure 7a). After 6 hours of culture (Figure 7b) the number of cells expressing PrPc at their surface appeared to increase, and after 48 hours 93% of tonsil lymphocytes expressed PrPc (Figure 7d).

Lymphocyte activation did not affect PrPc expression

In contrast to resting blood lymphocytes, 20% of the lymphocytes freshly isolated from tonsils were activated and expressed cell surface activation molecules such as CD25, receptor for IL-2, or CD69.

To verify whether PrPc expression is directly correlated to cell activation status, we activated peripheral lymphocytes using PMA+ ionomycin. After 48h of culture 60% of the lymphocytes were stained by CD25 or CD69. However the measured PrPc expression (Figure 8c) was the same as that on freshly isolated blood cells or control cells cultured in the medium alone (Figure 8b). Similarly, tonsil lymphocytes maintained in the same culture conditions as blood cells, 48 hours with (Figure 8c) or without mitogens (Figure 8b), appeared to increase their PrPc expression with increased duration of culture.

Peripheral blood lymphocytes

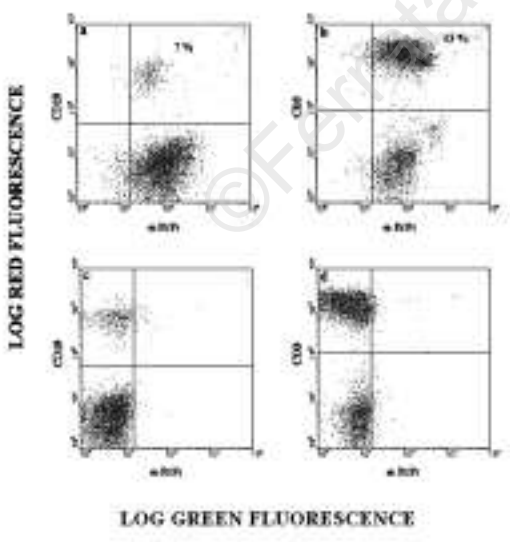

Figure 3. Two-color flow cytometry of peripheral blood B (a) or T (b) lymphocytes. Lymphocytes were stained with the 8G8 Mab (green fluorescence) and with the B marker anti-CD19 (red fluorescence) or with the T cell marker anti-CD3 (red fluorescence). For controls (c-d), PrPc-specific 8G8 antibody was omitted.

Tonsil lymphocytes

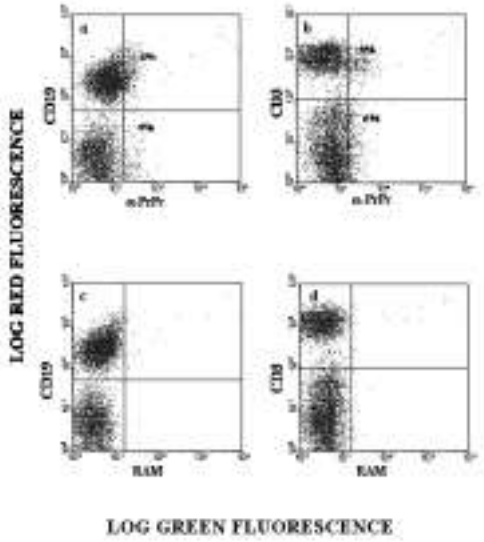

Figure 4. Two-color flow cytometry of tonsil B (a) or T (b) lymphocytes. Lymphocytes were stained with the 8G8 antibody (green fluorescence) and with the B-cell marker anti-CD19 (red fluorescence) (a) or with the T cell marker anti-CD3 (b). For controls (c-d), PrPc-specific 8G8 antibody was omitted.

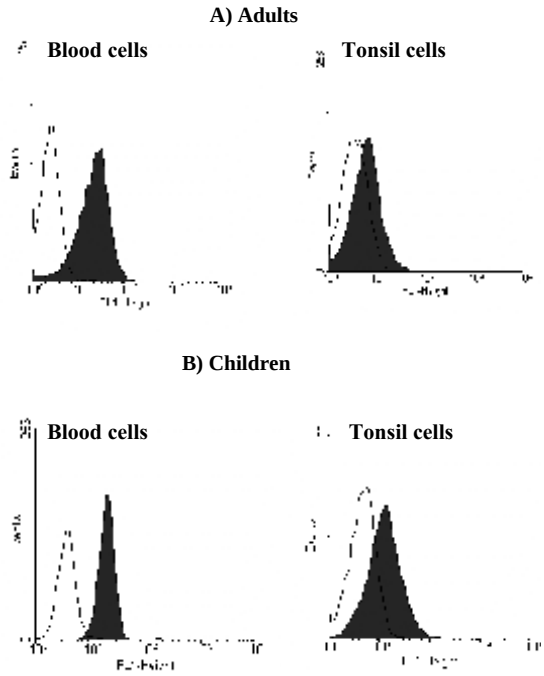

Figure 5. Comparative histograms of PrPc lymphocyte expression between peripheral blood cells and tonsil lymphocytes stained indirectly with PrP-specific 8G8 antibody (closed histograms) and rabbit anti-mouse antibody conjugated to fluorescein isothiocyanate or secondary antibody alone (open histograms). A: Children; B: Adults.

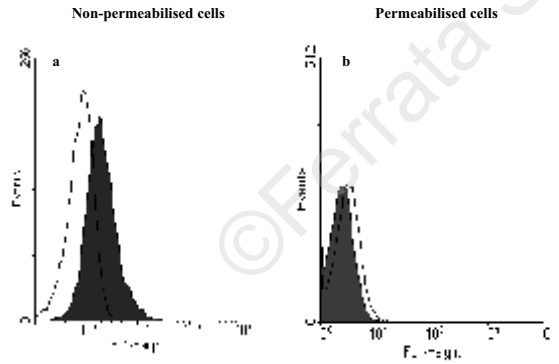

Figure 6. Tonsil lymphocyte permeabilization did not enhance PrPc expression. Fluorescence histograms of PrPc expression on tonsillar lymphocytes before (a) and after permeabilization (b).

Figure 8 (on the right). PrPc expression on tonsil cells is not increased by cell activation. PrPc expression was tested on freshly isolated cells (a), cells incubated for 48h in culture medium (b) or activated for 48 hours (c). Flow cytometry histograms of surface PrPc expression (closed histograms) are similar to those of non-activated blood or tonsillar lymphoid cells after 48 hours of culture (b) or activation (c).

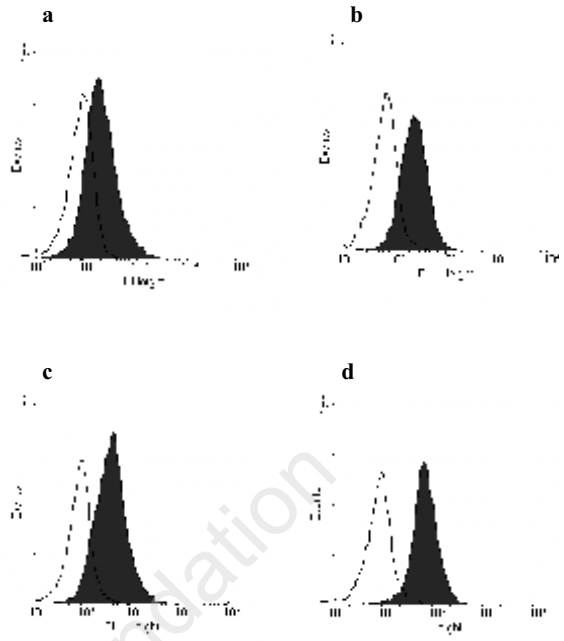

Figure 7. Tonsil lymphocyte surface PrPc expression is increased by cell culture, PrP-positive fluorescence histograms (closed histograms) from freshly isolated lymphocytes (a) or lymphocytes cultured in the medium alone, without mitogen, for 6 hours (b), 24 hours (c) and 48 hours (d). Open histograms represent the control staining (RAM-FITC).

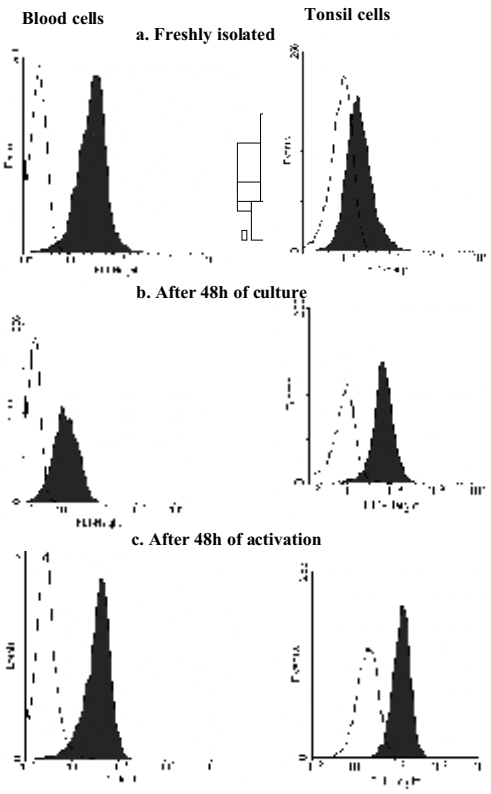

Table 1. Expression of PrPc on resting or activated human blood and tonsil lymphocytes.

Lymphocytes	*Blood (n=12)*		*Tonsil (n=12)*	
	Activated cells (%)	*PrPc⁺ cells (%)*	*Activated cells (%)*	*PrPc⁺ cells (%)*
Freshly isolated	0	99±4	20±4	15±9
After 48h of culture	0	90	33±10	96±3
After 48h of activation	60±6	93±3	90±5	98±43

These experiments suggest that all human lymphocytes express PrPc to a variable extent on their membranes and that this expression is not increased by activation (data summarized in Table 1).

Discussion

Immune cells[17,20] appear to play an important role in scrapie neuroinvasion following peripheral inoculation. However the nature of the cells involved remains unclear.

In mouse scrapie, prion infection can be transmitted by injection of cells from peripheral lymph organs. PrPc must be expressed on the cells of the host animal, since lymph organs of $PrP^{-/-}$ mice cannot transmit prion infection, but the protein need not be expressed by the recipient's B-cells.[21] In this study, we used cytofluorometry to analyze expression of PrPc on human blood lymphocytes and tonsil lymphocytes. As previously described,[9,22] we observed PrP protein at the surface of resting human B- and T-blood lymphocytes and both B- and T-lymphoblasts (data not shown). We did not distinguish any PrP-negative subpopulations among these mature cells. However, in accordance with previous data suggesting the absence of PrPc expression during granulocyte differentiation,[23] we did not detect PrPc on granulocytes or erythrocytes.

However, we observed that PrPc is less expressed on the surface of lymphocytes isolated from tonsil than on blood lymphocytes. Since freshly isolated tonsil cell populations contain about 20% activated cells, as shown by CD25 or CD69 expression, one may hypothesize that PrPc expression might be reduced by cell stimulation. This suggestion is, however, in conflict with others reports indicating that PrPc expression is significantly enhanced following *in vitro* stimulation with concanavalin A, a non-specific T-cell mitogen.[24] To clarify this issue, we used cytometry to measure PrPc expression on lymphocytes prepared from blood and tonsils, and cultured in presence of two mitogens, concanavalin A and phorbol myristate acetate with added ionomycin.

After only 6 hours of culture in the presence of the mitogens, the great majority of blood B- and T-cells became activated, as evidenced by the increase in cell size and the appearance of the activation markers CD25 and CD69. Yet the measured expression of PrPc on these activated B- and T-lymphocytes was about the same as that on freshly isolated, and hence resting, blood cells. Sometimes PrPc expression seemed to diminish in control cells cultured in medium alone. This loss is probably due to the appearance of apoptotic lymphocytes, since many lymphoid cells in culture, when not stimulated, undergo programmed cell death.

These data indicate that *in vitro* activation does not affect PrPc expression on either B- or T-cells. The percentage of cells expressing PrPc was the same in mitogen-activated or control cells cultured in medium alone. The mean fluorescence intensity increased, however, after even a few hours of culture, whether a mitogen was present or not, indicating that increased PrPc expression was the result of culture and not of activation. Thus, in tonsils, the low level of PrPc expression may result from a reduced PrPc synthesis that could be enhanced in stressful situations but may also indicate a steady detachment of PrPc caused by interactions with other cells or by enzymatic cleavage in lymphoid organs. Internalization does not apparently explain the low surface PrPc expression since permeabilization of the cells did not enhance the fluorescence signal in cytometry.

Our results may suggest that PrPc expression is lost by lymphocytes during intratonsillar migration or that PrPc expression is downregulated in lymphoid organs. In order to enter the tonsils, B- and T-cells must pass through endothelial venules, migrate, and make connections with matrix components. These adhesion mechanisms require dynamic membrane events that may induce changes in PrPc expression. Whether PrPc plays a role in intracellular communication at this location, as it appears to do in the nervous system, remains an open question.

In conclusion, human peripheral lymphocytes, normal tonsil lymphoid cells, human T- and B-cell lines, but not erythrocytes or granulocytes, express PrPc on their surfaces.

Our data suggest that cell activation does not affect PrPc expression, contrary to previous findings on human blood cells[9] and mouse splenocytes.[24] We suggest that the quantity of PrPc on the surface of lymphoid cells may be related to the cell's environment (blood, lymphoid tissues) or be modified by interactions occurring, for instance, during diapedesis through venules or during cell-to-cell contacts.

Further study is required to verify this hypothesis and also to compare the abilities of blood and tonsillar lymphoid cells to transfer infection.

Contributions and Acknowledgments

EH designed the study and NA co-ordinated the group. OJ, BC,WZ analyzed the data . NA wrote the manuscript, which was revised by EH and JYC. The prion protein antibodies were kindly provided by the Deutsche Primatenzentrum GMH represented by Prof. G. Hunsmann.

NA signs as first author because she designed the experiments and wrote the manuscript; JYC designed the study and revised the manuscript; BC did the different cytometric analyses; OJ purified the granulocytes and helped in the analysis of the data; WZ established the collaboration with the group of JYC. EH, the director of the Service of the Human Histology laboratory, supervised the research and revised the manuscript.

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Disclosures

Conflict of interest: none.

Redundant publications: no substantial overlapping with previous papers.

Manuscript processing

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Potential Implications for clinical practice

- These results confirm the actual risk of prion disease transmission from human blood components.

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