Glycosylation-related gene expression profiling in the brain and spleen of scrapie-affected mouse

Florence Guillerme-Bosselut2, Lionel Forestier2, Chantal Jayat-Vignoles3, Jean-Luc Villette4, Iuliana Popa5, Jacques Portoukalian5, Annick LeDur7, Hubert Laude7, Raymond Julien2, and Paul-François Gallet1,2

1INRA, UMR 1061 Génétique Moléculaire Animale – Université de Limoges, F-87060 Limoges, France; 2INRA, UMR 6101 Physiologie Moléculaire de la Réponse Immune et des Lymphoproliférations – Université de Limoges, F-87025 Limoges, France; 3CNRS, UMR 6101 Physiologie Moléculaire de la Réponse Immune et des Lymphoproliférations – Université de Limoges, F-87025 Limoges, France; 4INRA, UR 1313 Génétique Animale et Biologie Intégrative, F-78350 Jouy-en-Josas, France; 5EA 4169 Université Lyon-1, Hopital Edouard Herriot, F-69000 Lyon, France; 6Institute of Macromolecular Chemistry, Iasi, Romania; and 7INRA, UR 892 Virologie Immunologie Moléculaires, F-78350 Jouy-en-Josas, France

Received on December 18, 2008; revised on April 17, 2009; accepted on April 18, 2009

A central event in the formation of infectious prions is the conformational change of a host-encoded glycoprotein, PrPC, into a pathogenic isoform, PrPSc. The molecular requirements for efficient PrP conversion remain unknown. Altered glycosylation has been linked to various pathologies and the N-glycans harbored by two prion protein isoforms are different. In order to search for glycosylation-related genes that could mark prion infection, we used a glycosylation-dedicated microarray that allowed the simultaneous analysis of the expression of 165 glycosylation-related genes encoding proteins of the glycosyltransferase, glycosidase, lectin, and sulfotransferase families to compare the gene expression profiles of normal and scrapie-infected mouse brain and spleen. Eight genes were found upregulated in “scraipie brain” at the final state of the disease. In the spleen, five genes presented a modified expression. Three were also upregulated in the spleen of infected mice, and two (Pigq and St3gal5) downregulated. All changes were confirmed by qPCR and biochemical analyses applied to Pigq and St3gal5 proteins.

Keywords: brain/gene expression/microarray/prion disease/spleen

Introduction

Transmissible spongiform encephalopathies (TSE), or prion diseases, constitute a group of related neurodegenerative disorders affecting both humans (Creutzfeldt–Jakob disease (CJD)) and animals (scrapie of sheep and goats, chronic wasting disease in deer and elk, and bovine spongiform encephalopathy (BSE)). These diseases are characterized by the accumulation of a protease-resistant isoform (PrPSc) of the host-encoded prion protein (PrPC) (Prusiner 1998), the mechanism underlying the transconformation process being still unclear.

Many genes have been suggested to contribute to prion replication through comparative analyses of brain gene expression of healthy and prion-infected hamster (Riemer et al. 2000), sheep (Cosseddu et al. 2007), and mouse (Dandoy-Dron et al. 1998; Kopacek et al. 2000; Xiang et al. 2004; Brown et al. 2005; Skinner et al. 2006; Sorensen et al. 2008), at the terminal stage of the disease. Among these identified genes, only a few were glycosylation-related genes (Hexb, Ext2, and Galnt2) (Kopacek et al. 2000; Sorensen et al. 2008), and the proteins they encode (hexosaminidase B (beta polypeptide), exostoses (multiple) 2, and UDP-N-acetyl-alpha-d-galactosamine:polypeptide N-acetylgalactosaminyltransferase 2, respectively) are not associated with the glycosylation metabolic changes that are described in prion diseases (Hounsell 2004).

The prion protein contains two conserved N-glycosylation sites that are not necessarily occupied (Endo et al. 1989). In Syrian hamster brain, each of the two N-glycosylation sites of PrPC and PrPSc contains more than 50 different glycan motifs (Rudd et al. 1999; Ritchie et al. 2002), including the potential recognition epitopes LewisX and sialyl LewisX. The major variation observed between PrPSc and PrPC is related to their antennary N-glycan levels. PrPSc contains a higher proportion of tri- and tetra-antennary complex-type glycans, suggesting a lower level of mannosyl (beta-1,4)-glycoprotein beta-1,4-N-acetylgalactosaminyltransferase (Mgat3) activity toward PrPC in some of the cells in which PrPSc is formed (Rudd et al. 1999; Stimson et al. 1999). This enzyme catalyzes the formation of a bisecting GlcNAc residue which prevents the formation of new antennae but not the elongation of preexisting antennae.

Brain and cerebrospinal fluid acetylcholinesterase (AChE) N-glycosylation is also found to be altered in CJD. The percentage of AChE bound to concavalin A is reduced (Silveyra et al. 2006) and the insulin receptor alpha- and beta-subunits harbor a modified N-glycosylation. In neuroblastoma cells (N2a) infected with scrapie prion, an overall reduction in the glycosylation of the insulin receptor is observed that negatively affects its affinity toward insulin (Nielsen et al. 2004).

There is growing evidence that the prion protein interacts with various glycosylated molecules. This protein, like many other glycosylphosphatidylinositol (GPI)-anchored proteins, is mainly located in rafts of the plasma membrane (Naslavsky et al. 1997) where it interacts not only with galactosylceramide (GalCer) and sphingomyelin (Klein et al. 1998) but also with the ganglioside GM3 (Mattei et al. 2002). At the cell surface, it also establishes strong interactions with sulfated glycosaminoglycans (GAGs) (Pan et al. 2002; Warner et al. 2002). The binding of PrPC with GAGs could increase its endocytosis and consequently reduce the PrPSc pool available at the cell surface for conversion (Shyng et al. 1995). GAGs were recently shown...
to interfere with the disease process, providing prospects for therapeutic approaches (Adjou et al. 2003).

To investigate the possible modifications of the glycosylation machinery and to search for glycosylation-related genes that could mark prion infection, we report the comparative expression profile of numerous glycosylation-related genes in the brain and spleen of healthy and prion-affected mice, as these two tissues contribute to prion replication (Aguzzi and Heikenwalder 2006) while having different glycomes (Comelli et al. 2006).

The cDNA microarray used allows the simultaneous measurement of the expression of 165 glycosylation-related genes encoding glycosyltransferases, glycosidases, lectins, and various carbohydrate active enzymes. Only a few genes were found to be misregulated in terminally ill scrapie-infected animals. An RT-PCR kinetic analysis of their expression revealed that they were upregulated in the brain at a late incubation stage while in the spleen, the St3gal5 and Pigq genes were first slightly upregulated postinoculation and then downregulated.

Results

Microarray analysis and qPCR validation of gene expression

We used the tg338 mouse line. This line is a well-established prion mouse model consisting of transgenic mice that overexpress the VRQ allotype of sheep PrP, possibly infected by the fast propagating natural sheep scrapie strain 127S (Laude et al. 2002). In this model, the first clinical signs occur around 60 days after intracerebral inoculation (dpi) and are shortly followed by the animal death. A typical PrP signal is observed before and after PK treatment, both in the brain and the spleen of 60 dpi scrapie-diseased mice. This demonstrates that the replication of the infectious agent occurs both in the nervous central and extraneural systems (Figure 1).

Gene expression patterns were determined by a transcriptomic approach using a focused microarray and purified mRNA from the whole brain and spleen of scrapie-infected and mock-inoculated tg338 mice at 60 dpi. Reproducibility was assessed by using three RNA samples isolated from three different scrapie-infected mice and three control RNA samples similarly isolated from different mock inoculated mice. We used the flip labeling procedure (dye-swapping or reverse labeling with Cy3 and Cy5 dyes) with separate chips to avoid any bias associated with unequal incorporation of the two Cy dyes into cDNA. Overall, a total of 18 expression data were obtained for each cDNA probe present on the microarray. Spots with relative fluorescence intensities showing at least a 2-fold variation between the scrapie and the control states were retained for further investigations.

Among the 165 glycosylation-related genes tested, only eight and five genes showed such a variation in the brain or in the spleen, respectively (Table 1). In the brain of the ill mice, the eight overexpressed genes belong to the glycosyltransferase (B3gal1; St6galnac6; Mga1; St3gal2; St6gal1; Galnt6; Lfng) and glycosidase (Hexb) families. In the spleen, five genes belong to the glycosyltransferase (St6gal1; St3gal5), glycosidase (Man2a1; Hexb), and glycosylphosphatidylinositol anchor synthesis (Pigq) families and were over- or underexpressed in the disease state. Two of these genes (St6gal1 and Hexb) were observed both in the brain and the spleen and were upregulated in the two infected tissues.

The differential expression of the genes selected through the microarray analysis was confirmed by real-time qPCR (Figure 2). For all the genes identified, except St3gal2, the mRNA relative differential level of expression was found to be higher when tested by qPCR, probably reflecting the differences in the accuracy and sensitivity between the two approaches.

Time-course analysis of the differentially expressed genes

To assess the relationship between the observed transcriptomic modifications and the disease development, a qPCR kinetic analysis of the differentially expressed genes identified was performed. A similar expression profile was observed for the eight genes in the brain that were only upregulated at the very late inoculation stage (Figure 3).

In the spleen, three (Hexb, Man2a1, and St6gal1) of the five identified genes presented a kinetic of expression similar to the ones observed for the brain genes (Figure 4). The two other genes (Pigq and St3gal5) shared together a slight overexpression at 15 or 30 dpi followed by a progressive but deep decrease until 60 dpi.

Biochemical analysis of Pigq and St3gal5 gene expression

We next assessed the correlation between these transcriptional alterations and the encoded-protein synthesis and activity.

The Pigq protein is a component of the complex involved in the first step of GPI anchor synthesis. Due to the difficulty in directly measuring the protein activity, we performed a Western blotting of the protein using a homemade antibody (Figure 5). The signal appeared as a single band of 70 kDa, the protein theoretical mass being 66.26 kDa. The Western-blot signal
Discussion

Despite the recognized pivotal role of the prion protein (Prusiner 1998), various data indicate that other host factors may be involved in the infection process and contribute to the prion replication (Diaz et al. 2005; Caughey and Baron 2006; Tamgune et al. 2008). Among these cofactors, not only host-derived molecules, such as polyanionic polymers like sulfated glycosaminoglycans, but also anionic glycolipids might be good candidates. Various data showed that the N-glycans of PrP\textsuperscript{Sc} are different from those of PrP\textsuperscript{C}. The modifications include the glycan motifs with higher levels of complex: tri- and tetra-antennary glycans in PrP\textsuperscript{Sc} (Rudd et al. 1999; Ritchie et al. 2002) and the glycosylation pattern of the protein (Russelakis-Carneiro et al. 2002). Moreover, glycosylation alterations of other cell glycoproteins (Silveira et al. 2006), and also of the extra-cellular matrix (Barret et al. 2005) were identified.

Given the various protein- and lipids-linked glycan functions, the modifications of the glycosylation machinery, and consequently of glycoconjugate structures or amount, are likely to occur during the prion disease development. To address this question, we analyzed the expression level of 165 glycosylation-related genes in the brain and spleen of scrapie- and mock-inoculated mice, two tissues primarily involved in prion replication (Aguzzi and Heikenwalder 2006).

Among the genes identified, two were upregulated in both the brain and the spleen of terminally ill scrapie-infected mice. One is Hexb, which encodes the \( \beta \) subunit of lysosomal hexosaminidases HexA and HexB. It is responsible for the degradation of GM2 gangliosides, and for a variety of other molecules containing terminal N-acetyl hexosamines. Interestingly, an overexpression of this gene was previously reported to occur in the brain of scrapie-infected mice and was correlated with an accumulation of PrP\textsuperscript{Sc} in the lysosomes at the late stage of the disease (McKinley et al. 1991; Laszlo et al. 1992; Kopacek et al. 2000). The second gene is St6gal1, a gene encoding an enzyme that mediates the transfer of sialic acid residue to type 2 (Galβ1-4GlcNAc) disaccharide (Harduin-Lepers et al. 2001). In rat and mouse brain tissues, 75% of N-glycans are acidic and half of them carry sialic acid as the only anionic charge (Rudd et al. 2001). The remainders carry additional charges harbored by sulfate, glucuronic acid, and phosphate. In contrast, in the prion protein, all charged glycans carry sialic acid as the only anionic charge (Rudd et al. 2001). Thus, it could be expected that St6gal1 gene increased expression would affect the prion protein glycosylation. However, the structural analyses of the glycan structures harbored by both PrP\textsuperscript{C} and PrP\textsuperscript{Sc} did not reveal changes in the protein sialylation (Rudd et al. 1999; Ritchie et al. 2002). The reasons behind this apparent discrepancy remain unknown.

A comparative analysis of PrP\textsuperscript{C} and PrP\textsuperscript{Sc} N-glycosylation showed that the antennary level of PrP\textsuperscript{Sc} N-glycans was higher with an increased proportion of tri- and tetra-antennary complex-type glycans, suggesting a lower level of N-acetylglcosaminyltransferase III (Mgat3) activity toward PrP\textsuperscript{C} (Rudd et al. 1999; Stimson et al. 1999). In the present study, we surprisingly observed an overexpression of the Mgat3 gene in scrapie-infected brains. The knockout of the Mgat3 gene in mice showed that it did not affect the development of the disease (Tamgune et al. 2008). The PrP\textsuperscript{Sc} conformation and accumulation occur in a subset of cells. It is possible that
Fig. 3. Time-course analysis of genes identified in the brain. The expression level of the genes was examined by qRT-PCR during the course of disease development. Values in ordinate represent the mRNA relative expression in the scrapie brains comparatively to the same gene in the healthy brains. The gray area on each panel represents modification of expression within a range of ±2 that was considered as not significant. Data are expressed as mean ± range of nine expression measurements.

the Mgat3 gene expression is only highly upregulated in these cells but that it has no direct effect on the speed of and clinical signatures associated with the development of the disease. It remains that the biological significance and origin of the reported modification of the PrPSc antennary level need to be clarified.

On the other hand, the observed expression alterations may not necessary be involved in the disease pathogenesis, but may reflect disease-associated processes, such as inflammation. In the advanced stages of the disease, a conspicuous atypical inflammatory response occurs with recruitment of mononuclear cells and microglia activation in the brain (Perry et al. 1995). Genes presenting an overexpression at the late stage of the disease could be involved in the pro-inflammatory process in this tissue. The β3galt5, Galnt6, St3gal1, and St6galnac6 gene products catalyze the synthesis of glycan structures involved in the inflammatory response (Lowe 2003; Jou et al. 2006). The Lfng gene encodes a glycosyltransferase that initiates the elongation of O-linked fucose residues attached to EGF-like repeats in the extracellular domain of Notch molecules. These molecules are not described as acting in the inflammatory process, but a Notch signaling pathway constitutes a signaling network that plays a role in tissue repair during inflammation (Katoh Y and Katoh M 2006) and was reported to be activated in the brain of humans and animals dying of prion disease (Ishikura et al. 2005).

A disease-related overexpression of the St6gal1 gene in the spleen has not previously been described. However, the proposed role of the extra-medullar-derived (e.g., in spleen) St6Gal1 protein in regulating inflammation might fit with this observation (Nasirikenari et al. 2006). The Man2a1 misregulation might affect various pathways as this protein controls conversion of high mannose to complex N-glycans present on glycoproteins, and as a consequence, its activity modulates the maturation of numerous glycoproteins.
Glycosylation-related gene expression in prion disease

Fig. 4. Time-course analysis of genes identified in the spleen. The expression level of the genes was examined as in Figure 3. The gray area on each panel represents modification of expression within a range of ±2 that was considered as not significant. Data are expressed as mean ± range of nine expression measurements.

*Pigq* and *St3gal5* show a particular expression profile. They are slightly overexpressed between 15 and 30 dpi and then highly underexpressed at the final stage. The Western-blot signal of the Pigq protein was in good agreement with the transcript amounts measured at 60 dpi. This protein stabilizes the enzyme essential in the first step of GPI biosynthesis (Hong et al. 1999) and is required for an efficient GPI biosynthesis (Tiede et al. 2001). Numerous functions have been attributed to the GPI anchors, including signal transduction and cellular communication, and GPI-anchored proteins have various biological roles (for a review see Paulick and Bertozzi (2008)). The *Pigq* gene expression is modified only in the spleen. This lymphoreticular tissue is a crucial compartment where the prions replicate efficiently before the neuroinvasion (Aguzzi and Heikenwalder 2006). The prion splenic replication occurs rapidly following infection (Inoue et al. 2005) and the early slight upregulation of the *Pigq* gene expression might be related to this event, bearing in mind that the replication-competent cells are minority members of this tissue. Since the following decrease in the *Pigq* mRNA level in infected spleen occurs well after the onset of PrPSc protein accumulation, it could reflect mild pathological alterations of this tissue, as observed in scrapie-diseased mouse and sheep (McGovern and Jeffrey 2007).

In patients with CJD (Ohtani et al. 1996) and in scrapie-infected hamsters (Di Martino et al. 1993), the ganglioside content was reduced in the *brain* from terminally ill subjects. We
observed a significant modification of St6galnac6 gene expression in the brain of terminally ill mice. The encoded protein is involved in the synthesis of alpha-series gangliosides and possesses an alpha-2,6-sialyltransferase activity toward GD1a, GT1b, and GM1b gangliosides (Tsuchida et al. 2003). These minor gangliosides are expressed in the nerve terminals of specific cholinergic fibers (Irie et al. 1994) and have been reported to potentiate the effects of nerve growth factors (Fusco et al. 1993). The observed St6galnac6 gene overexpression could result from an activation of a NGF response to compensate the brain neuronal loss at the final stage of the disease. Interestingly, measurements of ganglioside differences in CJD-affected brain tissues revealed severe decreases in GD1a and GT1b (Ando et al. 1984; Ohtani et al. 1996). The gangliosides GD1a and GT1b are substrates for the St6galnac6 protein, and their decreased amount may be explained by the observed elevated St6galnac6 gene transcription level.

As described in the brain (Di Martino et al. 1993; Ohtani et al. 1996), we detected a slight but significant decrease of the ganglioside content in the spleen from terminally ill mice. Among all the genes involved in ganglioside biosynthesis, only the St3gal5 gene expression was downregulated. The corresponding St3gal5 activity and the GM3 ganglioside content were decreased (Table III). The highly decreased activity of the GM3 synthase may be explained by a reduction of the enzyme amount and consequently of an alteration of its molecular organization. The St3gal5 enzyme is organized in a hetero-multimeric structure corresponding to the association of β4galnt6, St3gal5, and St8 sia5 enzymes (Giraudo and Maccioni 2003). The sub-Golgi localization of the glycosyltransferase complex is modified according to the relative expression of these enzymes revealing an adaptation of the topology of the glycolipid

Fig. 5. Pigq and TfIId immunodetection in spleen of mock and scrapie-inoculated mice. Tissue homogenates were prepared at 60 dpi. Protein samples (25 μg) were resolved with a 10% SDS–PAGE, and the specific proteins were visualized by immunoblotting using anti-Pigq and anti-TfIId antibodies as primary antibodies, and alkaline phosphatase-coupled secondary antibodies as described in Material and Methods. The TfIId protein signal was indeed unchanged (A), the TfIId protein signal was indeed unchanged (B).

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Gene name</th>
<th>Fold change</th>
<th>Encoded protein</th>
<th>Protein function</th>
</tr>
</thead>
<tbody>
<tr>
<td>Brain</td>
<td>B3gal5</td>
<td>4.0 ± 1.5</td>
<td>Beta-1,3-galactosyltransferase 5</td>
<td>Glycosyltransferase—catalyzes the transfer of Gal to GlcNAc-based acceptors. Synthesis of O-glycans and glycolipids</td>
</tr>
<tr>
<td></td>
<td>St6galnac6</td>
<td>3.6 ± 1.5</td>
<td>Alpha-N-acetylgalactosaminide alpha-2,6-sialyltransferase 6</td>
<td>Glycosyltransferase—synthesis of alpha-series gangliosides</td>
</tr>
<tr>
<td></td>
<td>Mgat3</td>
<td>3.5 ± 0.5</td>
<td>Beta-1,4-mannosylglycoprotein 4-beta-N-acetylglucosaminyltransferase</td>
<td>Glycosytransferase—catalyzes the transfer of GlcNAc in beta 1–4 linkage to the mannose of the trimannosyl core of N-linked sugar chains</td>
</tr>
<tr>
<td></td>
<td>St3gal2</td>
<td>3.4 ± 1.4</td>
<td>CMP-N-acetylneuraminate-beta-galactosamide-alpha-2,3-sialyltransferase</td>
<td>Glycosyltransferase—catalyzes alpha-2,3 linkage of NeuAc to Gal-beta-1,3-GalNAc found in certain glycoproteins, oligosaccharides and glycolipids</td>
</tr>
<tr>
<td></td>
<td>St6gal1</td>
<td>3.1 ± 0.5</td>
<td>Beta-galactoside alpha-2,6-sialyltransferase 1</td>
<td>Glycosyltransferase—catalyzes the transfer of NeuAc to galactose containing acceptor substrates</td>
</tr>
<tr>
<td></td>
<td>Hexb</td>
<td>2.9 ± 0.5</td>
<td>Beta-hexosaminidase subunit beta</td>
<td>Glycosidase—catalyzes the degradation of GM2 gangliosides and other molecules containing terminal N-acetyl hexosamines</td>
</tr>
<tr>
<td></td>
<td>Galnt6</td>
<td>2.4 ± 0.2</td>
<td>UDP-N-acetyl-alpha-D-galactosamine:polypeptide N-acetylgalactosaminyltransferase 6</td>
<td>Glycosyltransferase—catalyzes the transfer of an N-acetyl-d-galactosamine residue to a serine or threonine residue on the protein receptor</td>
</tr>
<tr>
<td></td>
<td>Lfng</td>
<td>2.2 ± 0.2</td>
<td>Beta-1,3-N-acetylglucosaminyltransferase lunatic fringe</td>
<td>Glycosyltransferase—catalyzes the transfer a GlcNAc to the fucose residue. Elongation of O-fucose of Notch molecules</td>
</tr>
<tr>
<td>Spleen</td>
<td>St6gal1</td>
<td>2.7 ± 1.4</td>
<td>Beta-galactoside alpha-2,6-sialyltransferase 1</td>
<td>Glycosyltransferase—catalyzes the transfer of NeuAc to galactose containing acceptor substrates</td>
</tr>
<tr>
<td></td>
<td>Man2a1</td>
<td>2.7 ± 1.1</td>
<td>Alpha-mannosidase 2</td>
<td>Glycosidase—controls the final hydrolytic step in the N-glycan maturation pathway</td>
</tr>
<tr>
<td></td>
<td>Hexb</td>
<td>2.6 ± 1.5</td>
<td>Beta-hexosaminidase subunit beta</td>
<td>Glycosidase—catalyzes the degradation of GM2 gangliosides and other molecules containing terminal N-acetyl hexosamines</td>
</tr>
<tr>
<td></td>
<td>Pigq</td>
<td>−3.2 ± 0.8</td>
<td>Phosphatidylinositol N-acetylgalactosaminyltransferase subunit Q</td>
<td>Glycosyltransferase—controls the transfer of Gln to phosphatidylinositol, first step of GPI biosynthesis</td>
</tr>
<tr>
<td></td>
<td>St3gal5</td>
<td>−6.7 ± 2.1</td>
<td>Lactosylceramide alpha-2,3-sialyltransferase</td>
<td>Glycosyltransferase—catalyzes the formation of ganglioside GM3</td>
</tr>
</tbody>
</table>

Values (mean ± s.d.) were determined from a total of 18 gene expression analyses.
Both at the protein (Stuermer et al. 2004) and lipid (Zech et al. 2009) levels, a diminution of the GM3 content could participate in this process.

A significant reduction in glucosylceramide amount (25.4%) is observed in scrapie-infected spleens. This glycolipid is the product of the glucosylceramide synthase, an enzyme encoded by the Ugcg gene (Ichikawa et al. 1996). As no modification of the expression level of this gene, but also of the other genes involved in the gangliosides metabolism (except St3gal5), was observed in the spleen (data not shown), the reduced amount of glucosylceramide may be attributed to modified catalytic activities of enzymes involved in synthesis or degradation of gangliosides at a posttranscriptional level. Inhibition of GM3 synthase in murine embryonic fibroblasts resulted in activation of an alternate pathway oriented to o-series gangliosides (GM1b) and to derivative gangliosides GalNac-GM1b and GD1α (Shevchuk et al. 2007). In the present situation, the significant reduction of glucosylceramide may be explained by the orientation of ganglioside synthesis to a similar pathway. Alternatively, the decreased amount of glucosylceramide could result from a modification of its catabolic pathway, such as a posttranscriptional activation of Gba2 as observed in human Gaucher spleen (Vaccaro et al. 1988).

In summary, the focused microarray allowed the identification of eight and five glycosylation-related genes in the brain and the spleen, respectively, of scrapie-infected mice. The deregulation of the genes in the brain occurred only at the final stage of the disease and may reflect the pro-inflammatory response of the tissue to the disease. This observation may also be valid for the late splenic St6gal1 upregulation. The two other genes found in the spleen encode proteins that could act directly on the prion protein anchorage (Pigg) or environment (St3gal5). The results also point out modifications of the splenic metabolism of glycosphingolipids associated with prion disease. More precise analyses might allow the identification of the subtype of cells into which they occur. Altogether, these results highlight that the brain and splenic glycosylation machineries are affected during prion diseases, suggesting their potential implication in TSE development.

### Material and methods

#### Biological models

The overexpressing homozygous mouse line used (tg338) is transgenic for the VRQ allele of ovine PrP and has been previously described (Vilotte et al. 2001; Laude et al. 2002).

Mice, 6- to 8-week-old females, were inoculated intracerebrally with 20 μL of a 10% (wt/vol) brain homogenate in 5% glucose. The 127S ovine strain, obtained by propagation in tgOv mice of a natural sheep scrapie isolate (Vilotte et al. 2001), was used as an infectious source. Using this strain, the first clinical symptoms of scrapie are observed around 60 dpi, a few days before death. Control mice were inoculated with a healthy sheep–brain material.

The gene expression analysis with the cDNA microarray was performed at 60 dpi. A time-point analysis was also performed at 15, 30, 45, and 60 dpi by qRT-PCR. For the two analyses, three scrapie-infected and three mock-inoculated mouse brains and spleens were used.

Total RNAs from the whole brain and spleen were isolated using the acid guanidinium thiocyanate–phenol chloroform extraction method.

### Table II. GM3 synthase activity (pmoles/mg protein/h) in spleen of mock inoculated and scrapie-infected mice

<table>
<thead>
<tr>
<th>Condition</th>
<th>Average percentage of reduction</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mock inoculated</td>
<td>4.6%</td>
</tr>
<tr>
<td>127S infected</td>
<td>6.0%</td>
</tr>
</tbody>
</table>

Data (mean ± s.d.) resulted from at least three independent activity measurements, each from one different spleen microsomal fraction.

### Table III. Gangliosides content of mock-inoculated and 127S-infected mouse spleen

<table>
<thead>
<tr>
<th>Gangliosides content</th>
<th>Mock inoculated</th>
<th>127S infected</th>
<th>t-test (P value)</th>
<th>Average percentage of reduction</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total gangliosides (ng lipidic sialic acid/mg prot.)</td>
<td>2262</td>
<td>2114</td>
<td>4.6%</td>
<td>6.0%</td>
</tr>
<tr>
<td>GM3 gangliosides (ng lipidic sialic acid/mg prot.)</td>
<td>464</td>
<td>412</td>
<td>4.5%</td>
<td>9.8%</td>
</tr>
<tr>
<td>GM3 gangliosides/total gangliosides (%)</td>
<td>20%</td>
<td>19.7%</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Glucosylceramides (ng/mg prot.)</td>
<td>1421</td>
<td>1075</td>
<td>0.3%</td>
<td>25.4%</td>
</tr>
</tbody>
</table>

Each value was obtained from analyses carried out on two distinct spleens.

### Table IV. Glucosylceramide synthase activity in spleen of mock inoculated and scrapie-infected mice

<table>
<thead>
<tr>
<th>Material and methods</th>
<th>Mock inoculated</th>
<th>127S infected</th>
<th>t-test (P value)</th>
<th>Average percentage of reduction</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucosylceramide synthase activity (pmoles/mg protein/h)</td>
<td>3.7</td>
<td>8.9</td>
<td>1.3</td>
<td>88.5%</td>
</tr>
</tbody>
</table>

Each value was obtained from analyses carried out on two distinct spleens.

### Table V. Glycosphingolipids content of mock-inoculated and 127S-infected mouse spleen

<table>
<thead>
<tr>
<th>Glycosphingolipids content</th>
<th>Mock inoculated</th>
<th>127S infected</th>
<th>t-test (P value)</th>
<th>Average percentage of reduction</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total glycosphingolipids (ng/mg prot.)</td>
<td>1.3</td>
<td>2.0</td>
<td>1.3</td>
<td>88.5%</td>
</tr>
<tr>
<td>Glucosylceramide (ng/mg prot.)</td>
<td>1408</td>
<td>1034</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>GM3 gangliosides (ng/mg prot.)</td>
<td>464</td>
<td>412</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>GM3 gangliosides/total gangliosides (%)</td>
<td>20%</td>
<td>19.7%</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Glucosylceramides (ng/mg prot.)</td>
<td>1421</td>
<td>1075</td>
<td>0.3%</td>
<td>25.4%</td>
</tr>
</tbody>
</table>

Each value was obtained from analyses carried out on two distinct spleens.
method and further purified according to the RNaseasy mini kit procedure (Qiagen Inc., Hilden, Germany). RNA quantity and quality were assessed using the Agilent 2100 Bioanalyzer (Agilent Technologies, Massy, France) and agarose gel electrophoresis.

cDNA microarray analysis and data acquisition

DNA microarrays were prepared on polylysine slides (CML, Menzel-glasses). They contained 165 hybridization units (each DNA fragment was 450–500 bp) divided as 100 hybridization units specific to glycosyltransferases, 30 to glycosylhydrolases, 3 to lectins, 20 to sulfotransferases, and 12 units specific to genes involved in GPI synthesis (see Supplementary data 1 for the list of genes). The slides also contained 23 control units corresponding to 10 normalization units (the list of genes). The slides also contained 3 to lectins, 20 to sulfotransferases, and 12 units specific to glycosyltransferases, 30 to glycosylhydrolases, DNA fragment was 450–500 bp) divided as 100 hybridization units (the list of genes). The slides also contained 23 control units corresponding to 10 normalization units (Arabidopsis genes), 7 elongation units, and 1 positive and 5 negative controls.

Each unit was present in triplicate. Labeled cDNA synthesis elongation units, and 1 positive and 5 negative controls. corresponding to 10 normalization units (the list of genes). The slides also contained 23 control units corresponding to 10 normalization units (Arabidopsis genes), 7 elongation units, and 1 positive and 5 negative controls. Each unit was present in triplicate. Labeled cDNA synthesis and microarray hybridizations were performed as described in the MICROMAX™ TSA™ labeling and detection kit (NEN-Perkin-Elmer, Courtaboeuf, France). Equal amounts of biotin-labeled cDNAs (corresponding to 10 μg of total RNA from mock-inoculated brain or spleen) and fluorescein-labeled cDNAs (corresponding to 10 μg of total RNA from scrapie-infected brain or spleen) were hybridized on the slide. Various amounts (1–50 μg) of control RNAs (Arabidopsis RNAs, SpotReport-10 Array Validation System from Stratagene, Amsterdam, the Netherlands) were added to each batch of RNA samples for normalization.

Hybridizations were carried out overnight at 65°C in a hybridization chamber (Corning, New York). After washings, biotin-labeled cDNAs were revealed by streptavidin horseradish peroxidase (HRP) and Cy5-tyramide. Fluorescein-labeled cDNAs were revealed using an anti-fluorescein-HP antibody and Cy3-tyramide. Cy3 and Cy5 fluorescence signals were measured using a GMS 418 Array Scanner (Affymetrix, Santa Clara, CA). The raw data were analyzed using the Array-Pro Analyzer software (Media Cybernetics, San Diego, CA).

For each RNA sample, three different labeling were performed with biotin- or fluorescein-labeled nucleotides. Hybridizations between scrapie-infected or mocked samples were done using a dye-swap scheme.

Quantitative RT-PCR analysis

Reverse transcription was performed at 42°C for 50 min with the superscript II RNase H⁻ reverse transcriptase (Invitrogen, Cergy Pontoise, France) using 1 μg of DNase treated total RNAs and 0.5 μg oligo-d(T) primers in the 50 μL reaction buffer and stopped by incubation at 70°C for 15 min.

Semiquantitative RT-PCR was performed in triplicate. The transcription factor IId (TfIId) was used as an internal control (Milhiet et al. 1998). Primers were designed using Primers Express™ (PE Applied Biosystems) and are listed in supplementary data 2.

Real-time quantitative RT-PCR reactions were performed on the ABI Prism™ 5700 sequence detection system (Applied Biosystems) using SYBR green (Power SYBR Green PCR Master Mix, Applied Biosystems) as recommended by the manufacturer using serial dilutions of cDNAs (1, 1:4, 1:16, 1:64, and 1:256). We ensured that the relationship between the threshold cycle (Ct) and the log[RNA] was linear (−3.5<slope<−3.2).

The amplification of a single size product was further verified by gel electrophoresis (data not shown).

Data are expressed as the gene transcript relative amount in the scrapie-infected versus mock-inoculated mice. For a detail procedure, refer to the web site docs. appliedbiosystems.com/pebiodocs/04303859.pdf.

Preparation of brain and spleen homogenates for prion protein immunodetection

The whole brain or spleen was homogenized twice using Ultra-Turrax T-25 (IKA, Lille, France) at 24,000 × rpm for 30 s in the ice-cold lysis buffer (Tris-HCl 50 mM, pH 7.5, 0.5% Triton X-100, 0.5% deoxycholate Na). After centrifugation at 1000 × g for 10 min at 4°C, the supernatant was stored in aliquots at −20°C. The protein amount in the fraction was measured using the Bradford procedure (Bradford 1976).

The PrPSc detection was carried out according to the standard procedures from 250 μg total proteins for brain or 1 mg total proteins for spleen homogenates. The primary antibody used for PrP detection in the brain was SAF-84. It recognizes the sheep protein sequence between amino acids 161–171. Because of the difficulty in detecting PrPSc in the spleen with an SAF-84 antibody, the 8G8 antibody which recognizes the sheep protein sequence between amino acids 96–111 was used in this tissue. The two antibodies were used at 100 ng/mL and purchased from Cayman Chemical (Ann Arbor, USA).

Preparation of spleen extract and Pigq and TfIId proteins immunodetection

For Pigq immunodetection, mice spleens (healthy and scrapie) were homogenized twice for 1 min at 24,000 × rpm at 4°C in 3 mL of Tris-HCl 50 mM, pH 7.5, glycerol 15% buffer, containing 10 μL/mL of cocktail protease inhibitor (Sigma-Aldrich) with an Ultra-Turrax T-25 (IKA). The tissue extract was centrifuged for 15 min at 13,000 × g and 4°C, and the supernatant was recovered and corresponded to the splenic crude microsomal fraction. Proteins were quantified by the Bradford micro-method procedure (Bradford 1976) and 25 μg micrograms of the extracts were resolved on 10% SDS–PAGE. The proteins were transferred onto nitrocellulose membranes (Amersham), and Western blots were performed as described in the BM Chemiluminescence Blotting Substrate (POD) protocol (Roche Diagnostics, Meylan, France). The primary and HRP-conjugated antibodies were used in the 0.5% blocking reagent solution. The incubation time was overnight at 4°C for the primary antibody and 30 min at 20°C for the secondary one (HRP-labeled). Reactive proteins were visualized with the BM Chemiluminescence Blotting Substrate (POD) kit (Roche Diagnostics).

The antibody used for Pigq detection was produced in a rabbit and directed against the mouse protein sequence FRNDQDERPVRLSHW191. For the Western blotting, a purified fraction of the rabbit serum was used at the dilution of 1/250. The secondary antibody used was goat anti-Rabbit IgG coupled to HRP at a dilution of 1/2000 (Dako).

For TfIId immunodetection, spleens were homogenized as previously described except that the buffer contained 1% SDS (w/v). The crude extract was centrifuged for 5 min at 3000 × g and 4°C to discard cellular debris, and the protein amount was determined in the supernatant. Fifty micrograms of proteins were resolved on 10% SDS–PAGE, and the TfIId protein was...
detected after transfer with a rabbit polyclonal anti-TfIIId antibody (Santa-Cruz Biotechnology, Heidelberg, Germany) at a dilution of 1/1000.

Spleen subcellular fractionation and sialyltransferase assays
The subcellular fractionation and the subsequent enzyme activity measurement were realized in triplicate. For each measurement, one healthy or scrapie mouse spleen was homogenized at 4°C in 3 mL of 0.25 M sucrose, 10 mM Tris-HCl, pH 7.4, buffer containing 10 μL/mL of a cocktail of the protease inhibitor (Sigma-Aldrich), with an Ultra-Turrax T-25 (IKA) for 1 min twice at 24,000 × rpm. The microsomal fraction was recovered by differential centrifugations according to Dong and Hart (1994).

The GM3 synthase activity was measured according to the procedure described by Kono et al. (1998) using lactosylceramide as an acceptor. The reaction products were applied on C-18 Sep-Pak cartridge columns (Waters Associates, Milford, MA) and eluted with methanol. [14C]NeuAc incorporated to lactosylceramide was quantified by scintillation counting.

Glycolipids extraction and quantitation
Two mouse spleens were separately cut into small pieces, homogenized in chloroform–methanol 1:1 (v/v) by ultrasonic treatment and incubated overnight at room temperature before filtering. The solvent was evaporated at 45°C in a rotary evaporator (Buchi, Flawil, Switzerland) and the lipids taken up as previously described (Bouchon et al. 1985). Gangliosides were recovered on copolymer columns (Popa et al. 2002), evaporated at 45°C under nitrogen stream, and taken up in chloroform–methanol 1:1 (v/v). The lipid-bound was assayed by the periodate–resorcinol method (Jourdian et al. 1971). Lipids of the lower partitioning phase, containing ceramides and neutral glycolipids, were separated on LC-NH2 columns (Bodennec et al. 2000).

Lipids were separated by thin-layer chromatography on HPTLC silica gel 60 plates (Merck, Darmstadt, Germany), along with known amounts of standards (gangliosides from human melanoma tumors and bovine brain, neutral glycolipids, ceramides). Gangliosides were migrated in a flat bottom chamber with chloroform–methanol–water 65:25:4 (by volume). For neutral glycolipids, the migration solvent was chloroform–methanol–water 65:25:4 (by volume), and for ceramides, a chloroform–methanol 1:1 (v/v) was used.

Following separation, gangliosides were detected by spraying HPTLC plates with the resorcinol–HCl reagent (Svennerholm 1963) and heating at 100°C for 5 min. The chemical detection of the neutral gangliosides was done by spraying with the orcinol–H2SO4 reagent and heating at 100°C for 5 min. Ceramides were visualized by spraying plates with the copper acetate reagent (Fewster et al. 1969) and heating at 150°C for 10 min.

The densitometric analysis of plates was done by scanning with a ChromatoScan CS-930 (Shimadzu, Kyoto, Japan). Amounts of neutral glycolipids were calculated from the corresponding peak areas using a linear curve established with known amounts of standard glycolipids migrated and visualized as described above.

Supplementary Data
Supplementary data for this article is available online at http://glycob.oxfordjournals.org/.

Funding
The Conseil Régional du Limousin, by INRA (Institut National de la Recherche Agronomique) and by grants from GIS Infection à Prion.

Acknowledgements
The authors wish to thank Daniel Petit and Dominique Rocha for careful reading of the manuscript.

Conflict of interest statement
None declared.

Abbreviations
ACH, acetylcholinesterase; CJD, Creutzfeldt–Jakob disease; GAGs, glycosaminoglycans; GPL, glycosylphosphatidylinositol; HRP, horseradish peroxidase; PrPSc, host-encoded protein; PrPSc+, protease-resistant isoform; TSE, transmissible spongiform encephalopathies.

References
Glycosylation-related gene expression in prion disease


