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OPINION

Prion transmission in blood and urine: what are the implications for recombinant and urinary-derived gonadotrophins?

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Evidence is emerging that suggests that the protease-resistant isoform (PrP^{sc}) of the normal cellular prion protein (PrP^{c}) can be detected in the blood and urine of animals and humans with transmissible spongiform encephalopathies (TSEs). The production of the human menopausal and recombinant gonadotrophin preparations for use in ovarian stimulation protocols in fertility treatment is one area where the pharmaceutical industry needs to be vigilant and take appropriate steps to ensure that the safety of such drugs remains as high as ever. The recombinant preparations utilize fetal calf serum or other animal sera or proteins as part of a culture medium during production. Human urinary-derived menotrophin preparations are exposed to the theoretical risk of infection from menopausal donors of urine. Nevertheless, the failure to demonstrate irrefutably infectivity following intracerebral inoculation with urine from TSE-infected hosts suggests that the risk associated with products derived from urine is merely theoretical. Despite the paucity of evidence to date and its relevance to the infectious spread of TSEs, it is important that robust measures are implemented to either remove or inactivate PrP^{sc} in order to minimize contamination. Validation of each production process is required to assess the likelihood of contamination.

Key words: gonadotrophin/prion/TSEs/urinary isoforms

Introduction

Transmissible spongiform encephalopathies (TSEs) are neurodegenerative diseases that have been described in animals for >200 years, particularly in sheep as the disease scrapie. The emergence of bovine spongiform encephalopathy (BSE) in the UK in the 1980s was attributed to contaminated foodstuffs containing infected nervous tissue from sheep. This cross-species infectivity was not confined to ungulates, and in 1996 a new variant of Creutzfeldt–Jakob Disease (nvCJD) was identified in humans, supposedly due to the transmission of BSE from infected cattle (Will *et al.*, 1996). Almost all cases of CJD are spontaneous, inherited or iatrogenic in nature. However, a small number of nvCJD cases with a distinctive neuropathology were reported in adolescents and young adults from the UK, and represent a cohort of patients not normally associated with the disease. Subsequent evidence of transmission of BSE to primates, and strain typing in mice, has helped to confirm that BSE is the agent responsible for the emergence of the nvCJD in humans (Lasmezas *et al.*, 1996; Bruce *et al.*, 1997).

TSEs are believed to be caused by a self-replicating proteinaceous infectious particle known as a prion (PrP^{sc}). These are pathogenic variants of normal healthy protein isoforms, referred to as cellular prion protein (PrP^{c}). Unlike sporadic or iatrogenic CJD, in nvCJD the infectious agent is distributed in the lymph nodes, spleen and tonsils as well as neural tissue, even in the preclinical phase (Ghani *et al.*, 2000). Speculation has arisen over the presence of abnormal prions in extraneural tissue in nvCJD, which may present an even greater risk of blood infectivity than patients with CJD (Foster, 2000). It is currently impossible to quantify the risk associated with BSE because of uncertainties regarding the virulence of the BSE strain and the protracted incubation period, which may last up to 30–40 years.

The unique characteristic of PrP^{sc} and its unpredictable nature makes the management and control of TSEs all the more difficult. Extra regulatory measures have been recommended concerning blood transfusions since there is a theoretical risk for transmission via plasma-derived products, especially when derived from the UK, which has experienced the highest number of nvCJD cases as a result of the BSE outbreak (Franklin, 1999; Holada *et al.*, 2000). Although PrP^{sc} infectivity has not been established experimentally, due in part to expectedly low titres in blood and the absence of a homologous

human model of infection, it is still imperative that the authorities exercise the utmost caution. There is also new evidence to suggest that a protease-resistant PrP^{sc} isoform exists in the urine of infected animals (Shaked *et al.*, 2001). Although this finding has yet to be confirmed, it does emphasise the point that all biopharmaceutical products manufactured using substances of animal or human origin carry a theoretical risk of TSE transmission, including pharmaceutical gonadotrophin products. In fact, transmission of PrP^{sc} has occurred during infertility treatment with gonadotrophins derived from human pituitary material, leading to documented mortality from CJD in women between 11 and 14 years after treatment (Cochius *et al.*, 1990, 1992; Dumble and Klein, 1992; Collins and Masters, 1996). It has been stated that 11 women have died from the disease (B.Lunenfeld, personal communication). Whilst it is well documented that in the past, pituitary-derived preparations were obtained from corpses, it is also important to note that all such products have now been removed from the market.

In order to determine the significance and relevance of PrP^{sc} transmission in these products it is important to conduct risk assessments. These will assess several aspects such as levels of infectivity in the initial source material, the purification steps used to manufacture the product and the daily intake of PrP^{sc} relative to other source materials.

The aim of this paper is to assess the evidence for the transmission of infectious PrP^{sc} in blood and urine, to relate this evidence to fertility treatment with the gonadotrophin preparations menotrophin and recombinant FSH, and to discuss those techniques designed to remove or inactivate PrP^{sc} from biological materials.

Characteristics of prions

The fact that PrP^{sc} is characterized by a lack of genetic material yet has the ability to be infectious makes it unique and also difficult to eradicate. Measures that would normally be effective against a virus or a bacterium are often inappropriate. PrP^{sc} is extremely heat resistant, requiring between 30 and 60 min at temperatures >130°C for inactivation. However, even this may be inadequate since autoclaving at 134–138°C for 60 min does not result in complete inactivation (Taylor *et al.*, 1994). Furthermore, PrP^{sc} remains infective after sterilizing levels of radiation, exposure to strong acids, non-polar organic solvents, cremation at 343–360°C, and passage through 0.1 μm filters (0.2 μm filters can remove bacteria) (Brown *et al.*, 1990). PrP^{sc} and associated infectivity is more susceptible when exposed to reagents that are known to destroy proteins, such as strong concentrations of detergents, chlorox (sodium hypochlorite) and sodium hydroxide. Properties such as insolubility in non-ionic detergents and partial resistance to proteinase K digestion have enabled methods of separation to be developed which can isolate PrP^{sc} from the normal soluble PrP^{c} isoform (MacGregor, 2001).

Can prions be transmitted by blood and urine?

Evidence for transmission from blood

Normal PrP^{c} is expressed on many different cell types, including blood cells, and may have functions in cell physiology which are as yet undefined (Vostal *et al.*, 2001). The location of PrP^{c} in blood cells or plasma may facilitate the spread of TSE infectivity from the blood of infected animals. However, there are currently no tests that are sufficiently sensitive to detect infectivity via PrP^{sc} levels in blood, although tests which may eventually make this possible are under development (Brown, 2001).

Although there is emerging evidence to suggest that PrP^{sc}-related infectivity can be transmitted from species to species, it is important to interpret the results with caution. Human and animal blood cells display significantly different levels of PrP^{c}, and so species differences must be considered when extrapolating results of rodent transmission to humans (Holada and Vostal, 2000).

A recent study in scrapie-infected mice demonstrated infectivity in the spleen before spreading to the brain via T and B lymphocytes (Weissmann *et al.*, 2001). The lymphocytes themselves did not exhibit infectivity, suggesting that the presence of PrP^{sc} in blood does not necessarily imply that it is infectious. This is supported by evidence that suggests proteinase K sensitive PrP^{c} can be identified on the surface of peripheral blood mononuclear cells in both normal and scrapie-infected sheep (Herrmann *et al.*, 2001). Although it has not been possible to identify the infectious agent in blood, infectivity can be transmitted via blood. Whole blood taken from a sheep during the symptom-free phase of an experimental BSE infection was shown to transmit infection to another sheep following transfusion (Houston *et al.*, 2000).

The plasma of mice infected with 301v mouse-adapted BSE was able to transmit infection when injected intracerebrally into healthy mice and provides further evidence that BSE-related agents can be found in the blood or plasma of infected animals (Taylor *et al.*, 2000).

Infectivity bioassays in mice indicated that approximately seven times more plasma and five times more buffy coat are required to transmit infection by the i.v. route compared with the intracerebral route. It was suggested that the lack of epidemiological evidence for the transmission of TSEs from blood in humans is due to the absence of plasma infectivity prior to the onset of clinical symptoms and low levels during the symptomatic stage of the disease. Other factors include the reduction of infectivity by plasma processing steps and the need for higher levels to trigger infection via the i.v. route compared with intracerebral route (Brown *et al.*, 1999).

Exogenous spiking experiments have suggested a minimal risk of acquiring CJD from plasma concentrates since the fractionation steps remove the cellular components of plasma which are associated with the majority of infections (Brown *et al.*, 1998).

Evidence for transmission from urine

The evidence for transmission from urine still needs to be confirmed; however, a recent study demonstrated that a protease-resistant urinary isoform, defined as $uPrP^{sc}$, can be prepared from the urine of hamsters, humans and cattle affected with TSE (Shaked *et al.*, 2001). However, the implications of this finding are still uncertain because the urinary isoform differs from the brain-derived PrP^{sc}. When hamsters were

inoculated with the urinary isoform they did not develop clinical symptoms even after prolonged incubation (270 days), despite the presence of the isoform in their urine. In contrast, hamsters inoculated with brain-derived PrP^{sc} developed clinical symptoms within 80 days and had the urinary isoform of PrP^{sc} in their urine. Some aspects of the methodology may have influenced the appearance of this particular isoform in urine. The study used dialysis to separate and purify the prion; however, this technique may have favoured the production of a soluble transient isoform of PrP^{sc} from normal PrP^{c}, leading to higher than expected levels. It is recommended that the study be repeated without the use of the dialysis technique to establish the presence of infectivity in urine containing $uPrP^{sc}$. There are also suggestions that certain anionic detergents (i.e. sarkosyl and sodium dodecyl sulphate) are capable of inducing β-sheet aggregates distinct from PrP^{sc} in terms of infectivity and protease resistance (Xiong *et al.*, 2001), which may have contributed to the formation of the $uPrP^{sc}$ isoform observed in the Shaked study (Shaked *et al*., 2001) which used similar conditions.

Xiong *et al.* were unable to separate fibrillar and amorphous prion aggregates, the latter of which formed the majority in their analysis (Xiong *et al*., 2001). This may explain why they were unable to demonstrate proteinase K resistance following detergent/dialysis treatment. By using ultracentrifugation they might have been able to discriminate between the two distinct types of prion. In contrast, Shaked *et al.* performed ultracentrifugation after dialysis, which enabled them to detect scrapie-associated fibril (SAF)-like prion aggregates (Shaked *et al*., 2001). Repeating both studies may determine the influence of ultracentrifugation and dialysis on the formation of proteinase K-resistant prion aggregates.

The transmission of CJD from species to species by urine was demonstrated after urine from a man with CJD was intracerebrally inoculated into mice. However, this result was not repeated when urine from another patient or from a number of infected animals was used (Tateishi, 1985). Furthermore, subsequent investigators have been unable to reproduce these findings and the relatively short incubation period observed in this single case (compared with the time following inoculation with infected brain) suggests that transmission was probably due to cross-contamination within the laboratory (Baron, 1999). Another early study detected murine CJD infectivity in many tissues including blood, but was unable to confirm any infectivity in urine (Kuroda *et al.*, 1983). Bioassays of tissue from BSE-infected cattle used to inoculate mice failed to demonstrate infectivity in urine (Scientific Steering Committee, 2002).

Regarding the transmission of infectivity in urinary-derived gonadotrophins, it is important to consider the fact that proteins are rarely excreted into urine. Normal 24 h protein levels are $<$150 mg in total (40% albumin, 15% globulins and 40% tissue proteins) and represent the low molecular weight proteins such as microalbumin and α-1-acid glycoprotein. The molecular weight threshold for protein excretion is ~40 kDa, which would be expected to exclude the insoluble aggregated PrP^{sc} isoform. However, it seems possible that PrP^{sc} may be present in blood in a non-aggregated form (at concentrations below the recent detection limit), which is subsequently concentrated in the kidneys leading to $uPrP^{sc}$. The extent of urinary excretion of PrP^{sc} is probably less than that proposed in the Shaked study. PrP^{sc} initially entered the urine as a non-aggregated, protease-resistant isoform, but dialysis appears to have contributed to the formation of a transient semi-aggregated $uPrP^{sc}$ isoform, which concentrates in the urine. The impact of dialysis may have led to higher than expected levels of $uPrP^{sc}$.

Another source of $uPrP^{sc}$ could originate from the kidney itself. The kidney contains relatively low levels of PrP^{c} (Moudjou *et al.*, 2001), so it is feasible that PrP^{c} derived from the kidney could contribute to the overall $uPrP^{sc}$ levels.

Diagnosing prion infection

Ideally it would be preferable to control the source material and to screen for the presence of PrP^{sc}. However, the very nature of TSEs means that sourcing and screening can only be reliably performed on a retrospective basis. Sourcing urine or blood from so-called safe BSE countries will not guarantee PrP^{sc}-free materials. The BSE status of countries is constantly being updated due to the continuing emergence of new cases in countries previously regarded as being unaffected. There are also limitations to screening since the diagnosis of TSE relies on post-mortem brain samples, although there are a number of diagnostic tools under development which may provide a useful means of identifying infection at earlier stages. One of the problems often encountered is the sensitivity of screening tests. Experimental rodent models suggest a minimum requirement for PrP^{sc} detection in blood buffy coat of $<$10 pg/ml. The correlation of this level to humans will be dependent on whether infectivity in human blood is lower or greater than rodent experimental models. Some screening tests are within range, so it is feasible that we will be able to detect PrP^{sc} at practical levels in TSE-infected patient's blood and possibly urine in the near future (Brown, 2001). It is also important to consider that increasing the sensitivity of a test will be characterized by a rise in the number of false positive results, which may need further validation. Infectivity can be measured by bioassay using either endpoint titration or incubation time assay; however, patterns of infectivity vary according to the method used. These differences can be attributed to variations in the size and number of PrP^{sc} aggregates (Masel and Jansen, 2001). In-vitro tests are only capable of identifying components that suggest infectivity.

Diagnostic tests used for the surveillance of TSEs currently rely on post-mortem validation by microscopic examination of brain tissue. The current batch of tests available are essentially bioassays of post-mortem tissue, which are time consuming and expensive. Analysis of post-mortem tissue can be performed using a range of techniques, including histopathological examination of the brain, SAF detection by electron microscopy, Western blotting, immunoassay and immunohistochemical detection. It is also important to validate these tests in order to confirm the feasibility, standardization, assay performance, validation criteria, specificity for a particular isoform and predictability of the assay, i.e. it must be able to distinguish between normal and diseased tissue. Although we still rely on post-mortem tests as a means of determining infectivity, there are a number of in-vitro surrogate tests that

Table I. Features of the European Community approved diagnostic tests for prions

Prionics check assay
100% accurate (no false positive or negatives)
Uses brain tissue (post-mortem test)
Western blot
Samples digested with proteinase K resulting in degradation of PrP^{c} but most PrP^{sc} resists complete degradation
Remaining proteins are separated by size using electrophoresis
Transfer to a membrane
Detection by chemiluminesence – prion specific antisera linked to an enzyme
Detects BSE in preclinical animals
8–10 h turnaround
This test is currently being adapted for blood tests for both BSE and CJD
CEA (Commissariat a L'Energie Atomique) BioRad (Platelia™)
100% accurate (no false positives or negatives)
Uses brain tissue
Sandwich ELISA Immunometric assay
Prions in bovine samples are digested with proteinase K, again only partial degradation with PrP^{sc}
Specific antibodies used to coat ELISA plates before sample is introduced and the amount of PrP^{sc} is quantified by second antibody linked to an enzyme to form a detectable product
4 h turn around
potential for detecting BSE in preclinical animals
Enfer Scientific test
100% accurate (no false positive or negatives)
Uses spinal cord tissue thus is post-mortem test
ELISA (like BioRad)
4 h turnaround
potential for testing BSE in preclinical animals

have been developed which measure levels of proteins known to be associated with TSEs. These surrogate diagnostic tests involve the determination of the 14-3-3 protein, which is located in the cerebrospinal fluid and appears to act as a marker of neuronal cell death (Muller *et al.*, 2000). However, this test is not specific to TSEs as increased levels of this protein are also associated with other diseases such as Alzheimer's. Another surrogate test relies on the detection of the β-protein S-100, in which elevated levels have been demonstrated in patients with genetic and sporadic CJD (Beekes *et al.*, 1999). However, animal models suggest S-100 is inappropriate for the preclinical detection of scrapie in hamsters, although it may provide a useful in-vitro test for the diagnosis of TSE in naturally or accidentally infected animals.

A number of reliable and sensitive diagnostic tests are now available for BSE-infected animals. Although they are all currently restricted to post-mortem investigation, these tests are being developed and adapted to test for infection in preclinical animals. There are three tests currently in use that have been approved by the European Community: Enfer, CEA-BioRad and Prionics. A detailed summary of each of these tests is outlined in Table I.

Although these tests lack sensitivity, they may provide a useful tool for screening animal tissue donors, e.g. the production of fetal calf serum (FCS). However, the low levels of PrP^{sc} present in extraneural tissue mean that the confirmation of infectivity still relies on the inoculation of tissue in the susceptible animal, followed by a post-mortem analysis, while other in-vitro tests based on the detection of PrP^{sc} and/or other related surrogate proteins can only suggest infectivity.

Purification of recombinant versus urinary-derived gonadotrophin

In order to maintain the highest quality of animal- and human-derived biological products, it is essential that the food and pharmaceutical industries implement appropriate regulatory measures. From the following discussion it is entirely likely that in the case of the gonadotrophin preparations, their current production and purification techniques are sufficient to eliminate the risk of prion infection. However, it is important to validate each stage of the process to determine not just whether PrP^{sc} is present, removed or inactivated, but also whether infectivity is being eradicated.

Human plasma is already widely used to produce coagulation factors, immunoglobulin preparations and albumins. In the case of gonadotrophins, animal serum features prominently in the production of recombinant gonadotrophin products, and human urine from menopausal women is the source of FSH and LH in the production of human menopausal gonadotrophin (hMG) and highly purified hMG. Therefore, the theoretical risk of infection, albeit negligible, extends throughout the whole spectrum of animal- and human-derived biological materials.

The regulatory authorities have already responded to the potential threat of infection from blood products used for vaccines and have issued guidelines. Currently any bovine-derived material used in the manufacture of a vaccine is regulated according to the Committee for Proprietary Medicinal Products (CPMP) and is continually updated in response to new findings. These measures include exclusion criteria for the source of the plasma, donor deferral criteria and quarantine/withdrawal policies (Committee for Proprietary Medicinal Products, 2001). Such measures are now a prerequisite for products derived from urine as regulatory authorities become aware of the potential for infection. However, the lack of infectivity currently demonstrated in urine is reflected by the CPMP′s decision to place urine in the lowest risk category (Committee for Proprietary Medicinal Products, 2001). In the case of the gonadotrophin preparations, both types of production will have to undergo risk assessment analysis at each stage of production. The biotechnology industry is mainly concerned with validating existing steps in the manufacturing process, which will also apply to the manufacturers of gonadotrophins.

The menotrophins are the established gonadotrophin preparation and have been in use for ~40 years. Their safety record is impeccable and to our knowledge there have been no reports of infectivity with TSEs during this time. The recently introduced highly purified menotrophin (Menopur; Ferring Pharmaceuticals), which does not contain any additional protein other than gonadotrophins, has recently been shown to be comparable with recombinant FSH in terms of safety and tolerability, reflecting the equivalence in purity between both preparations (European and Israeli Study Group on highly purified hMG versus rFSH, 2002). FSH and LH activity are extracted from the urine of post-menopausal women from Argentina, which is known to be a low risk country for BSE and CJD infection. This is a medically well-controlled and

fairly static population of donors, which allows for the close monitoring of infectious materials.

However, there are limitations to screening and quarantine as a rapid means of identifying infection, which increases the emphasis on ensuring that there are robust removal or inactivation processes in place.

There at least two potential steps in the recombinant production process that could introduce contamination: FCS, which also contains cell debris and serum proteins, is used as a medium for culturing recombinant cells, and monoclonal antibodies, used in the purification process, which are derived from lymphocytic cells raised in tissue culture medium containing fetal bovine serum.

FCS is derived from a broad population of animals which, theoretically, is likely to be less well protected than humans from the risk of prion infection. The collection of FCS is a rather crude process, which permits the potential for transmission of infectivity from mother to fetus. The use of a bolt to the brain to slaughter the mother disrupts the blood brain barrier allowing the entry of PrP^{sc} into the systemic route. Furthermore, the physical removal of the calf can cause disruption to the placental barrier allowing contamination.

The purification stage using monoclonal antibodies could represent a significant risk for contamination since it is one of the final steps in the production process and the affinity columns cannot be sanitized afterwards to ensure the inactivation of residual infectious materials because the expensive monoclonal antibodies in the column would be destroyed.

In order to validate each step of the manufacturing process for both urinary-derived and recombinant gonadotrophins, the use of a spiking agent which mimics prion proteins is required. The principle of spiking involves a known amount of impurity, which closely resembles the agent of interest, being introduced in a sample to assess the capacity of the purification process to remove or inactivate prions. In the case of PrP^{sc}, it is difficult to obtain a pure sample and this may affect validation.

Several plasma protein purification steps have been investigated in plasma spiked with TSE-infected material. Western blot analyses of PrP^{sc} and bioassay measurements of infectivity revealed that the purification steps were effective at removing PrP^{sc} and associated infectivity (Lee *et al.*, 2001). In support of these findings, 16 plasma fractionation steps spiked with hamster-adapted scrapie showed that the most effective removal techniques included cold ethanol precipitation and depth filtration for albumin and immunoglobulin processes, and ion exchange columns used for the preparation of factors VIII and IX. Hence, the majority of steps are capable of removing abnormal PrP^{sc} (Foster *et al.*, 2000)

Urine-derived preparations

The purification steps in the production of highly purified hMG involve an initial stage of separation of the gonadotrophin hormones from the urine sample followed by four stages of chromatography to maximize purity (see Figure 1). Validation of each separation technique will be important and may well indicate which are already sufficient to remove PrP^{sc} and associated infectivity.

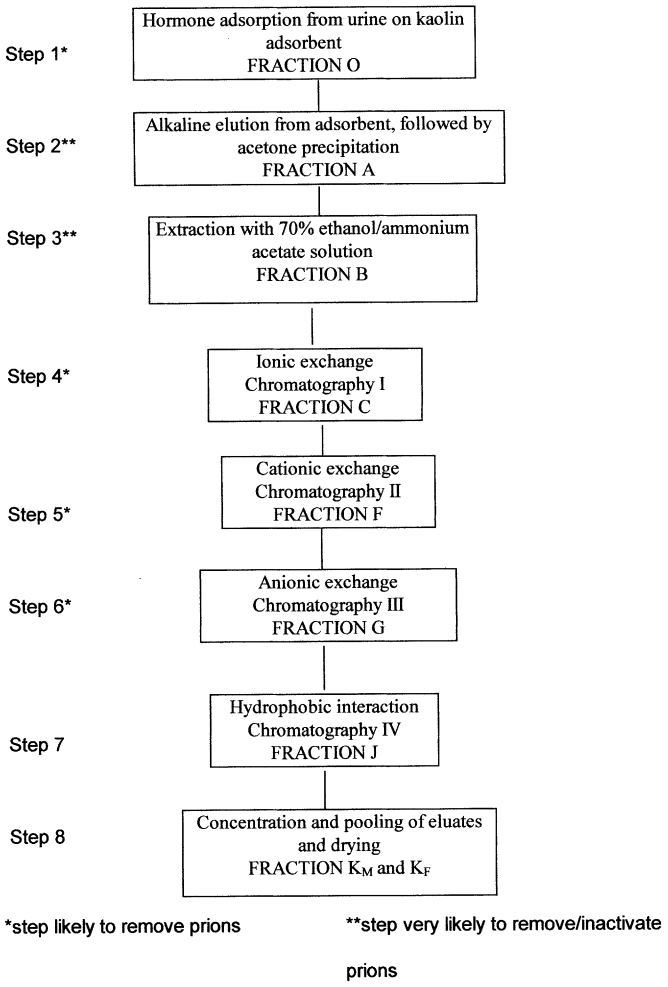

Figure 1. Purification process for highly purified menotrophins (Data on file, 2001). *Step likely to remove prions. **Step very likely to remove/inactivate prions.

The primary adsorption step using kaolin is likely to contribute to the removal of PrP^{sc}, especially as the desired compounds are eluted under alkaline conditions, which are known to inactivate prion infectivity. The eluate is precipitated by acetone, and the hormones are extracted in a solution containing 70% ethanol. PrP^{sc} is removed from plasma by ethanol precipitation with 20% (v/v) ethanol, so it is unlikely that it will be solubilized by 70% ethanol and therefore it is highly unlikely that PrP^{sc} will remain in the eluate after filtration.

Experience obtained in numerous validation studies, although mainly with plasma as a matrix, indicates that ion exchange chromatography (both cationic and anionic exchangers of any type), but not hydrophobic interaction chromatography, tends to remove PrP^{sc} and infectivity. Therefore three of the four chromatographic steps leading to highly purified gonadotrophin preparations from urine have the potential to remove PrP^{sc}. However, all of these steps require thorough validation studies with different types of spiking material.

Given the extremely low possible titre of infectivity in the starting material and the large number of potentially potent

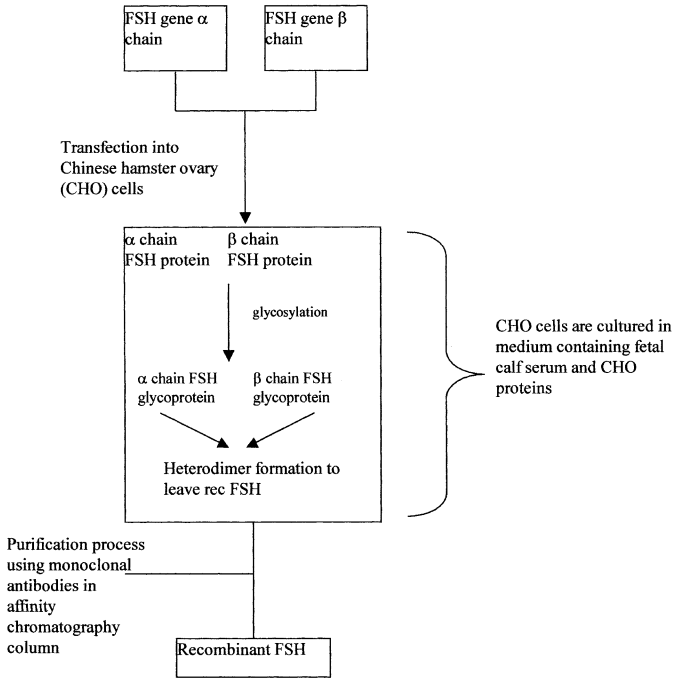

Figure 2. Production process for the recombinant proteins.

removal/inactivation steps, the products derived from urine can—pending the results of validation studies—be considered safe.

Recombinant preparations

The only source of possible contamination with PrP^{sc} is the bovine serum used for freezing and maintaining the recombinant Chinese hamster ovary cell lines and the hybridoma cell lines for producing the monoclonal antibodies used in purification (affinity chromatography), the production media for the recombinant hormones and the recombinant antibodies (see Figure 2). As outlined previously, the risk of contamination with PrP^{sc} is extremely low. Nevertheless, validation studies should be carried out to demonstrate the potential of PrP^{sc} removal in the purification steps. This is of particular importance, as the possible introduction of PrP^{sc} through the monoclonal antibodies used in affinity chromatography purification comes at a late stage of downstream processing.

If some of the purification steps used for the gonadotrophins are limited in their ability to remove PrP^{sc} there are alternative techniques available, such as depth filtration, which are proven to be effective and are inexpensive. It is worth noting that most of the effective purification steps involve the removal but not the inactivation of PrP^{sc}.

One of the more promising techniques that has already demonstrated considerable success is the Haemosafe method used for the inactivation of non-lipid-coated viruses, e.g. polio, and prions (Reichl, 1991). This process can be adapted to treat plasma and serum and is expected to generate similar results with urine. The main steps involve heating the sample in the presence of anionic tensides and stabilizers, which is then followed by incubation with chaotrophic reagents, e.g. urea, which can be conveniently included into existing purification schemes without the need for major investment.

Initial experiments using the Haemosafe technique have already demonstrated the recovery of 83% of hCG in a bioassay (Data on file, 1997). By carefully optimizing the two major steps, even higher recoveries can be achieved. The optimized process will again need validation studies to prove inactivation of PrP^{sc} infectivity.

During the design of validation experiments it is important to not only test the level of inactivation in each step but also to perform an overall validation of the entire process (Reichl *et al.*, 2002). It is also imperative that every manufacturer conducts their own specific validation assessment instead of assuming that a generic production process that has been validated by one manufacturer will apply to another.

Discussion

Although nvCJD is a serious condition it is still extremely rare. The scientific community as a whole is still only beginning to come to terms with the implications of TSEs and the potential impact on products sourced from animals. However, they are confident that providing the appropriate measures are taken at each stage of the manufacturing process, whether it is the pharmaceutical or the food industry, then the exposure to PrP^{sc} will be negligible.

In relation to the gonadotrophins, the method of production, whether it is extraction from urine or recombinant DNA technology, still utilizes material with a biological origin. PrP^{sc} is now known to cross the species barrier and furthermore is able to infiltrate and propagate in extraneural tissues. While there is growing evidence to suggest that infectivity can be transmitted in blood, the presence of PrP^{sc} in urine and associated infectivity is yet to be confirmed.

In experimental models to date, the unconfirmed infectivity of urine or blood can be explained as either subclinical infection, whereby the animals are merely carriers of the disease, which may or may not be able to transmit infection, or there is a considerably delayed onset of action compared with brain-derived PrP^{sc}.

Risk assessments need to be conducted in both blood- and urine-derived gonadotrophins to determine the actual likelihood of transmitting infection in ovarian stimulation protocols. The relative risk of these products is expected to be very low compared, for example, with the ingestion of meat products. Potential levels of exposure need to be considered and in the case of the gonadotrophin products very small volumes of drug are administered during one treatment cycle, hence the volume and frequency of exposure compared with food intake is negligible. Another factor to consider is the size of the batch used to derive the final product. If infectious PrP^{sc} from a donor is pooled into a much larger volume of urine or plasma then this will be diluted significantly, reducing the chances of exposure. Therefore, when we consider the daily intake of food compared with the comparatively negligible amounts administered during fertility treatment and the purification

processes utilized in the production of gonadotrophin preparations, we can conclude that the relative risk of infection is unlikely to be more than theoretical.

There are a number of techniques available to remove or inactivate PrP^{sc} throughout the manufacturing processes, which are applicable to the production of hMG and rFSH. Furthermore, diagnostic tests are under development, which will hopefully be able to rapidly identify preclinical TSEs using blood and urine samples. There are already stringent regulatory measures in place in order to account for viruses and bacteria during the manufacture of gonadotrophins and it is reasonable to assume that such steps are already capable of removing significant amounts, if not all, of PrP^{sc} during the manufacturing process.

Conclusion

It is imperative that manufacturers validate each stage of purification to ensure the highest levels of safety are maintained for gonadotrophins, irrespective of the original source of hormone and the manufacturing process used. The opportunity has arrived for existing procedures to be validated and tested using the most stringent criteria and, where necessary, there are additional removal/inactivation techniques available that will ensure that TSEs remain a theoretical and insignificant risk. Provided the pharmaceutical and food industries remain vigilant and flexible in the methods they use to regulate and manufacture their products, the threat of TSEs will fail to materialize.

References

Baron, H. (1999). In Prusiner, S.B. (ed). *Prion Biology and Disease.* Cold Spring Harbor Lab Press, USA, Chapter 17.

Beekes, M., Otto, M., Wiltfang, J., Bahn, E., Poser, S. and Baier, M. (1999) Late increase of serum S100 beta protein levels in hamsters after oral or intraperitoneal infection with scrapie. *J. Infect. Dis.*, **180**, 518–520.

Brown, P. (2001) Creutzfeldt–Jakob disease: blood infectivity and screening tests. *Semin. Hematol. supp.*, **38**, 2–6.

Brown, P., Liberski, P.P., Wolff, A. and Gajdusek, D.C. (1990) Resistance of scrapie infectivity to steam autoclaving after formaldehyde fixation and limited survival after ashing at 360 degrees: practical and theoretical implications. *J. Infect Dis.*, **161**, 467–472.

Brown, P., Rohwer, R.G., Dunstan, B.C., MacAuley, C., Gajdusek, D.C. and Drohan, W.N. (1998) The distribution of infectivity in blood components and plasma derivatives in experimental models of transmissible spongiform encephalopathy. *Transfusion*, **38**, 810–816.

Brown, P., Cervenakova, L., McShane, L.M., Barber, P., Rubenstein, R. and Drohan, W.N. (1999) 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. *Transfusion*, **39**, 1169–1178.

Bruce, M.E., Will, R.G., Ironside, J.W., McConnell, I., Drummond, D., Suttie, A., McCardle, L., Chree, A., Hope, J., Birkett, C. *et al.* (1997) Transmissions to mice indicate that 'new variant' CJD is caused by the BSE agent. *Nature*, **389**, 498–501.

Cochius, J.I., Burns, R.J., Blumbergs, P.C., Mack, K. and Alderman, C.P. (1990) Creutzfeldt–Jakob disease in a recipient of human pituitary-derived gonadotrophin. *Aust. NZ J. Med.*, **20**, 592–593.

Cochius, J.I., Hyman, N. and Esiri, M.M. (1992) Creutzfeldt–Jakob disease in a recipient of human pituitary-derived gonadotrophin: a second case. *J. Neurol. Neurosurg. Psychiatry*, **55**, 1094–1095.

Collins, S. and Masters, C.L. (1996) Iatrogenic and zoonotic Creutzfeldt–Jakob disease: the Australian perspective. *Med. J. Aust.*, **164**, 598–602.

Committee for Proprietary Medicinal Products (2001) Note for guidance for minimizing the risk of transmitting animal spongiform encephalopathy agents via human and veterinary medicinal products (February, **2001**, EMEA/401/01 – Final).

Data on file (1997) Ferring GmbH, Kiel, Germany.

Data on file (2001) Instituto Massone S.A. Arias 4431 (1430), Buenos Aires, Argentina.

Dumble, L.J. and Klein, R.D. (1992) Creutzfeldt–Jakob legacy for Australian women treated with human pituitary gonadotrophins. *Lancet*, **340**, 847–848.

European and Israeli Study Group on highly purified hMG versus rFSH (2002) Efficacy and safety of highly purified menotropin versus recombinant follicle-stimulating hormone in *in vitro* fertilization/ intracytoplasmic sperm injection cycles: a randomized, comparative trial. *Fertil. Steril.*, **78**, 520–528.

Foster, P.R. (2000) Prions and blood products. *Ann. Med.*, **32**, 501–513.

Foster, P.R., Welch, A.G., McClean, C., Griffin, B.D., Hardy, J.C., Bartley, A., MacDonald, S. and Bailey, A.C. (2000) Studies on the removal of abnormal prion protein by processes used in the manufacture of human plasma products. *Vox Sang.*, **78**, 86–95.

Franklin, I.M. (1999) The impact on British blood services on BSE and v-CJD: implications for patients, donors and public health. *Scott. Med. J.*, **44**, 35–36.

Ghani, A.C., Ferguson, N.M., Donnelly, C.A. and Anderson, R.M. (2000) Predicted vCJD mortality in Great Britain. *Nature*, **406**, 583–585.

Herrmann, L.M., Davis, W.C., Knowles, D.P., Wardrop, K.J., Sy, M.S., Gambetti, P. and O'Rourke, K.I. (2001) Cellular prion protein is expressed on peripheral blood mononuclear cells but not platelets of normal and scrapie-infected sheep. *Haematologica*, **86**, 146–153.

Holada, K. and Vostal, J.G. (2000) Different levels of prion protein (PrPc) expression on hamster mouse and human blood cells. *Br. J. Haematol.*, **110**, 472–480.

Holada, K., Simak, J. and Vostal, J.G. (2000) Transmission of BSE by blood transfusion. *Lancet*, **356**, 999–1000.

Houston, F., Foster, J.D., Chong, A., Hunter, N. and Bostock, C.J. (2000) Transmission of BSE by blood transfusion in sheep. *Lancet*, **356**, 999–1000.

Kuroda, Y., Gibbs, C.J. Jr, Amyx, H.L. and Gajdusek, D.C. (1983) Creutzfeldt–Jakob disease in mice: persistent viremia and preferential replication of virus in low-density lymphocytes. *Infect. Immun.*, **41**, 154–161

Lasmezas, C.I., Deslys, J.P., Demaimay, R., Adjou, K.T., Lamoury, F., Dormont, D., Robain, O., Ironside, J. and Hauw, J.J. (1996) BSE transmission to macaques. *Nature*, **381**, 743–744.

Lee, D.C., Stenland, C.J., Miller, J.L.C., Cai, K., Ford, E.K., Gilligan, K.J., Hartwell, R.C., Terry, J.C., Rubenstein, R., Fournel, M. *et al.* (2001) A direct relationship between the partitioning of the pathogenic prion protein and transmissible spongiform encephalopathy infectivity during the purification of plasma proteins. *Transfusion*, **41**, 449–455.

MacGregor, I. (2001) Prion protein and developments in its detection. *Transfus. Med.*, **11**, 3–14.

Masel, J. and Jansen, V.A.A. (2001) The measured level of prion infectivity varies in a predictable way according to the aggregation state of the infectious agent. *Biochem. Biophys Acta*, **1535**, 164–173.

Moudjou, M., Frobert, Y., Grassi, J. and La Bonnardiere, C. (2001) Cellular prion protein status in sheep: tissue-specific biochemical signatures. *J. Gen. Virol.*, **82**, 2017–2024.

Muller, W.E., Laplanche, J.L., Ushijima, H. and Schroder, H.C. (2000). Novel approaches in diagnosis and therapy of Creutzfeldt–Jakob disease. *Mech. Ageing Dev.*, **116**, 193–218.

Reichl, H.E. (1991) European patent 0530173.

Reichl, H.E., Foster, P.R., Welch, A.G., Li, Q., MacGregor, I.R., Somerville, R.A., Fernie, K., Steele, P.J. and Taylor, M. (2002) Studies on the removal of a BSE-derived agent by processes used in the manufacture of human immunoglobulin. *Vox Sang.* (in press).

Scientific Steering Committee (2002) TSE infectivity distribution in ruminant tissues. *European Commission Report*, January 10–11, 2002.

Shaked, G.M., Shaked, Y., Kariv-Inbal, Z., Halimi, M., Avraham, I. and Gabizon, R. (2001) A protease resistant prion protein isoform is present in urine of animals and humans affected with prion diseases. *J. Biol. Chem.*, **276**, 31479–31482.

Tateishi, J. (1985) Transmission of Creutzfeldt–Jakob disease from human blood and urine into mice. *Lancet*, **ii**, 1074.

Taylor, D.M., Fraser, H., McConnell, I., Brown, D.A., Brown, K.L., Lamza, K.A. and Smith, G.R. (1994) Decontamination studies with the agents of bovine spongiform encephalopathy and scrapie. *Arch. Virol.*, **139**, 313–326.

Taylor, D.M., Fernie, K., Reichl, H.E. and Somerville, R.A. (2000) Infectivity in the blood of mice with a BSE-derived agent. *J. Hosp. Infect.*, **46**, 78–79.

Vostal, J.G., Holada, K. and Simak, J. (2001) Expression of cellular prion protein on blood cells: potential functions in cell physiology and pathophysiology of transmissible spongiform encephalopathy diseases. *Transfus. Med. Rev.*, **15**, 268–281.

Weissmann, C., Raeber, A.J., Montrasio, F., Hegyi, I., Frigg, R., Klein, M.A. and Aguzzi, A. (2001) Prions and the lymphoreticular system. *Philos. Trans. R.. Soc. Lond. B. Biol. Sci.*, **356**, 177–184.

Will, R.G., Ironside, J.W., Zeidler, M., Cousens, S.N., Estibeiro, K., Alperovitch, A., Poser, S., Pocchiari, M., Hofman, A. and Smith, P.G. (1996) A new variant of Creutzfeldt–Jakob disease in the UK. *Lancet*, **347**, 921–925.

Xiong, L-W., Raymond, L.D., Hayes, S.F., Raymond, G.J. and Caughey, B. (2001) Conformational change, aggregation and fibril formation induced by detergent treatments of cellular prion protein. *J. Neurochem.*, **79**, 669–678.