



Scientific Steering Committee

OPINION
ORAL EXPOSURE OF HUMANS TO THE BSE AGENT:
INFECTIVE DOSE AND SPECIES BARRIER

ADOPTED BY THE SCIENTIFIC STEERING COMMITTEE
AT ITS MEETING OF 13-14 APRIL 2000
FOLLOWING A PUBLIC CONSULTATION VIA INTERNET BETWEEN
6 AND 27 MARCH 2000

The Scientific Steering Committee (SSC) adopted at its meeting of 2-3 March 2000 a preliminary opinion on *Oral exposure of humans to the BSE agent: infective dose and species barrier*. The document was put on Internet on 6 March 2000 and the international scientific community was invited comment on its contents and to propose a "realistic" worst case scenario for the assessment of the possible residual risk in bovine derived products.

Only two comments have been received by the secretariat of the SSC:

- From Dr. M.A. Smits, Dr. A. Bossers, Dr. J.P.M.Langeveld and Dr.Bram E.C.Schreuder, from ID-DLO, -Lelystad (The Netherlands)
- From Dr.Tim Miles, Veterinary Manager, Meat and Livestock Commission (United Kingdom)

These comments were discussed by the SSC at its meeting of 13-14 April 2000 and amendments in the pre-opinions were introduced, as shown in the revised opinion and report hereafter (**bold text**).

The SSC noted that, in its comments, the Meat and Livestock Commission (MLC) refers to cattle to cattle transmission studies on brain material obtained from affected cattle which have now been completed in the UK. The results will be published later this year. These studies seem to show that actual titres in cattle are significantly lower than those calculated by the Committee and it is therefore suggested that more reliance should be placed on these actual titres and less on the calculation of the titre in the Opinion.

The SSC invites the MLC and the research institute(s) involved in this research to communicate these results to the Committee. It will then, if appropriate, amend the attached opinion.

EXECUTIVE SUMMARY:

An analysis of the risk to humans of the entry of BSE infected tissues into the food chain needs to take account of the amount of the BSE agent needed to induce an infection in man. The occurrence of vCJD in humans is now accepted as an outcome of such an infection but there is no evidence which allows any direct assessment of the minimum amount of infective material needed to induce a human infection. Therefore a variety of different observations and experiments had to be used to come to a judgement about the likely minimum infective dose.

The amount of the BSE agent needed to infect cattle orally remains uncertain but in careful feeding studies less than one gram of brain tissue taken from a cow with clinical TSE induced disease in all the recipient cows and each gram may theoretically contain anything from 10 to a 1000 infectious units. This therefore highlights the need to assess the species barrier between cattle and man.

In the analysis conducted by the SSC's working party and *ad hoc* Group, the range of different TSEs have been assessed from a molecular, cellular, experimental animal and epidemiological point of view. Some species are susceptible to particular TSEs whereas others are not and the presence of a species barrier may be absolute or require a higher infective load before the recipient species becomes infected. The agents of BSE, vCJD and scrapie have been assessed experimentally in different species, in specially developed transgenic animals and in vitro cellular studies. Unfortunately, several of the crucial experiments have not yet been conducted or cannot be done for obvious reasons but there is so far no basis to infer that in practice a definite species barrier exists between cattle and man. There may be a barrier or there may be none (e.g. from 1 to 10,000), **although available evidence indicates that values greater than 1 are likely to be more realistic.**

The SSC has been unable precisely to identify a minimum dose of BSE infectivity required to cause infection in man.

The SSC considers that numerical combinations of the various worst case values (for example: dose + species barrier) for risk assessment purposes into one "multi-worst case figure" would eventually result in an unrealistic scenario. It therefore recommends that, *realistic* worst case scenarios are proposed by the international scientific community.

OPINION

Because a small amount of infected tissue can contain an infectious oral dose of the BSE agent for cattle and sheep (less or equal to 1g of homogenised infected brain tissue), concern has been raised on the limits of the human exposure dose which could theoretically establish an infection. The Scientific Steering Committee reviewed existing scientific data in order to produce an estimate of the order of magnitude of the oral human infectious dose.

A working group was established in the framework of the activities of the TSE/BSE *ad hoc* Group of the Scientific Steering Committee, with the following mandate:

1. Produce a document summarising the state of the art in relevant scientific disciplines;
2. Produce an estimate of the order of magnitude of the oral human infectious dose based on the most accurate scientific data available.

On the basis of the report of the Working Group, attached to this opinion, and the outcome of the discussions held by the TSE/BSE *ad hoc* Group, the SSC considers that the present opinion should be seen as completing the analysis made in its opinion on *Human Exposure Risk* adopted on 9-10 December 1999. **In the latter opinion, the measures that would prevent human exposure are extensively reviewed.** The complements in the present opinion can be summarised as follows:

- 1) It is impossible to produce an estimate of the order of magnitude of the human oral infectious dose. The available knowledge basis continues to be incomplete. *For example*, on the issue of species barrier, little new data became available in the last few years permitting the quantification of the size of the barrier of the cattle to humans transmission and of the possible additivity of exposure to low doses. Further research needs to be encouraged especially in fields such as (i) the physical nature of the infectious agent, (ii) the biological basis of the species barrier and (iii) the pathogenesis of TSE diseases upon oral exposure.
- 2) Quantitative assessment of the risks must take into account the size of the species barrier between cattle and humans, the infection source, the dose of agent, the estimation of the

minimum infectious dose and the potential effects of cumulative exposure to low doses of infected materials, the route of exposure and its efficacy in establishing TSE agent infection, the pathogenesis of TSEs and the genetic susceptibility.

- It is probable that *PRNP* gene is of paramount importance for the determination of the size of the cattle to human species barrier; this size is not known but the Scientific Steering Committee recommends that for risk assessments of human exposure to potentially BSE contaminated products, the assumption of a worst case scenario¹ considering no (=1) barrier **should be included**. In risk assessments, the range from 1 to 10.000 should be considered **although available evidence indicates that values greater than 1 are likely to be more realistic**. The **worst case scenario** would imply that the minimal infectious dose value(s) considered/accepted to be valid for intraspecies transmission of BSE would also apply for humans.
- Exposure to tissues from species known to be "naturally" infected by BSE agent is critical and the factor of most significance is likely to be the dose although no predictions can be made of the outcome as there are major uncertainties about the infectiveness of animal-derived TSE agents for humans. As repeatedly stated in several of the SSC opinions, tissues known to harbour high infectivity are to be excluded human and animal food chain in all regions where there is a BSE risk.
- The minimum infectious dose of BSE agent for humans is not known. There are several possible approaches to the calculation of the relative efficiency of different routes of infection, and hence to calculate an estimate of the effective titre of cattle tissue orally delivered to humans. Overall these various approaches yield highest estimated values from 10^1 to 10^3 ID₅₀/g cattle oral doses from a brain from a clinically affected animal. The higher value may represent a worst case scenario if the oral route is more efficient than experimental data suggest and if a particularly high

¹ Worst case scenarios are used as a tool in risk assessment. They are often based on an extrapolation of experiments and in most cases not representative of what happens under natural conditions. For example, laboratory experiments where it is the intention is to infect animals, are not necessarily representative for natural conditions of a human or animal eating food that is possibly infected. Experiments may be done on sensitive animal species and strains, whereas in reality, the sensitivity of a human population may be different and variable. The efficiency of transfer under natural conditions is also not known.

titre of infected brain is sampled. The possibility that such a high dose might be encountered is unlikely but cannot be ruled out. The SSC recommends that, in risk assessments, the range from 10 to 1000 ID₅₀/g cattle oral doses is considered.

On the other hand, risks from exposure to amounts of infection below the minimal infectious dose cannot be determined in the current state of the scientific knowledge. In terms of risk assessment, the risks resulting from (low) residual infectivity should at present - until further evidence is available - be calculated as if a population as a whole is exposed the SSC suggests using the probability scenario in the BSE context, assuming for the time being a linear dose-response curve down to the low dose range². This is a conservative assumption and would mean, as an example, that a product containing an evenly distributed residual infectivity of 10⁻³ ID₅₀/g and given to each of 1 million individuals, may result in 500 individuals being infected³. The SSC also suggests that the accumulative infectivity scenario has to be considered as valid for risk assessment of the effects of repeated sub-infectious doses, provided the interval of administration is not too long.

- The route of infection is critical: it is generally believed that patients with vCJD have been orally exposed to the BSE agent. Oral route is known to be the least effective in animal TSE models. However, a trans-species oral route cannot be assumed, to be less efficient, although nothing is known on the efficacy of inter-species transmission by oral route especially for natural diseases. A worst case assumes a value of 10⁸ cow i.c. ID₅₀ per gram of CNS tissue. It is estimated that there is a 10⁵ fold reduction in efficiency from i.c. to oral BSE transmission within one species, thus resulting in a worst case scenario of 10³ cow oral ID₅₀/g CNS tissue. However this *order of magnitude* is usually lower when a species barrier is crossed.

² The dose response relation is not known. Whether the dose/response relationship in the low dose range (for low levels of potential residual infectivity in products after appropriate processing and handling, i.e., after appropriate sourcing, removal of SRMs, processing, avoidance of cross-contamination, etc.) is linear or follows for example a sublinear dose-response relationship, does not immediately affect the outcome of the assessment as such (in terms of absolute numbers of people at risk) but may affect the perception of the risk in management terms.(See report)

³ This is just an example; elements such as the weight of the inoculum and of the infectious material or the weight of the bovine material to which the individual was exposed to., are not taken into account.

- Pathogenesis of human TSEs, including vCJD is unknown. However, data obtained from experimental TSE in animal models and from vCJD indicate the lymphoid organs as main target of TSE agents after peripheral infection. Infection of cells of the lymphoreticular system appears critical for neuroinvasion. Pathogenesis of BSE in cattle may not be identical.
 - Amongst possible other pre-disposing factors, genetic background is an important co-factor; today, only patients homozygous for Methionine/Methionine at codon 129 of the *PRNP* gene have developed vCJD. This does not preclude that individuals with other genotype at codon 129 will not develop disease in the future.
- 3) The SSC considers that numerical combinations of the various worst case values (for example: dose + species barrier) for risk assessment purposes into one “multi-worst case figure” would eventually result in an unrealistic scenario. It therefore recommends that, *realistic* worst case scenarios are proposed by the international scientific community.

REPORT FROM THE WORKING GROUP

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I. MANDATE AND SUMMARY CONCLUSIONS

Because a small amount of tissue can contain an infectious oral dose of the BSE agent for cattle⁴ and sheep⁵ (less or equal to 1g of homogenised infected brain tissue⁶), concern has been raised on the limits of the human exposure dose which could theoretically establish an infection. A working group was therefore established in the framework of the activities of the TSE/BSE *ad hoc* Group of the Scientific Steering Committee, with the following mandate:

1. Produce a document summarising the state of the art in relevant scientific disciplines;
2. Produce an estimate of the order of magnitude of the oral human infectious dose based on the most accurate scientific data available.

The SSC endorses the summary conclusions of the Working Group.

The state of the art is presented in the chapters II through VII of the present report. Each of these chapters contains also a section with a summary of the conclusions the SSC considers being important. The emphasis is on oral exposure. However, in practice, parenteral exposures e.g. from contaminated medicines, needle-sticks, conjunctival (e.g. in the abattoir) etc., should not be neglected. Experimentally, such exposures are dose for dose more likely to produce an infection than oral exposure as the latter is relatively inefficient.

The SSC concludes that, on the basis of currently available scientific knowledge relating to TSEs, it is impossible to produce an estimate of the order of magnitude of the human oral infectious dose. However, there is sufficient knowledge to suggest that the following elements contribute to the quantitative assessment of the risks that may result from the exposure of humans to products containing TSE infectivity:

1. **Infection source.** It is currently not possible to predict the outcome of known challenge or exposure of any species, including man, with a TSE agent based on historical knowledge, phylogenic classification or as judged by the *PrP* gene sequence or PrP analysis. Some orders and families of animals, notably *Ruminantia* and *Felidae*, appear to be more likely than others to succumb to field exposures of the scrapie or BSE agents. Despite this, even within these orders and families some genera or species appear to have a natural resistance.

In general, for humans, the greatest risk is from human-adapted strains of TSE agent. To date the only animal TSE agent that might be a human pathogen is the BSE agent from cattle. It is a new agent from a species previously devoid of known TSE disease. Risks to man from CWD and any new TSE agent cannot be assessed but may not be zero.

The critical factors that determine whether or not infection with the BSE agent would cause disease in an exposed human depend upon the strain of the agent, the infectivity titre, the efficiency of the route of infection, and the interactions of the agent with the host (including the PrP genotype of the host). Thus at the point where a human is

⁴ Bradley, 1996.

⁵ Foster, Hope and Fraser, 1993.

⁶ Pooled brain tissue, from cattle in which advanced clinical signs of BSE were diagnosed, inoculated orally in mice. The titration is still in progress.

exposed to the BSE agent, even if all the factors were known, the one of most significance is likely to be the dose but even then no predictions can be made of the outcome as there are major uncertainties about the infectiveness of animal-derived TSE agents for humans.

2. **Infection route.** Assuming that cattle tissues infected with the BSE agent are the cause of vCJD, although the route of entry has not yet been firmly established, it is generally believed that these patients have been orally exposed. Parenteral routes of exposure at primary (cattle to man) transmission are less likely for the general population but are possible for individuals in occupations that enable exposure to high risk tissues without adequate protection. In this situation, lower doses could result in disease and incubation periods could be shorter. Therefore, with comparable doses, a higher proportion of individuals exposed by parenteral routes could succumb compared with those exposed orally.

By contrast, oral exposure at secondary (man to man) transmission is unlikely. Parenteral exposure, however, is more likely as a result of surgical or medicinal treatment. This probability increases if there is a high prevalence of infected individuals in the general population, particularly since there is a lack of methods to identify them. This is because vCJD is characterised by prominent deposition of PrP^{Sc} in lymphoid tissues in the clinical phase of disease. This, combined with the finding of PrP^{Sc} in the appendix of one vCJD patient before the onset of clinical signs, suggests a potentially different pathogenesis from other human TSEs. It supports concerns about a potential man-to-man transmission of vCJD through surgery, contamination with blood, blood products and tissue grafts derived from persons harbouring vCJD infectivity.

3. **Efficacy of route of infection.** The most efficient route of TSE infection is by intracerebral injection, when the donor and recipient are from the same species. The available data suggest that titres in some parts of cattle brains could be as high as 10^8 cow i.c. ID₅₀/g.⁷ The relative efficiency of the oral route within species and between species is not known and a trans-species oral route cannot be assumed to be less efficient. A worst case assumes a value of 10^8 cow i.c. ID₅₀ per gram of CNS tissue. From animal model data, it is estimated that there is a 10^5 fold reduction in efficiency from i.c. to oral BSE transmission within one species. However, this order of magnitude is usually lower when a species barrier is crossed.
4. **Homozygosity.** All vCJD patients tested to date are homozygous for methionine (met) at codon 129 of the PrP gene, arguing that met homozygous individuals have the shortest incubation period and/or are more susceptible to the disease. It cannot be excluded that also valine/valine or heterozygous subjects may be of similar or lower susceptibility and will develop vCJD, but eventually with a longer incubation period.⁸ This is what happened in iatrogenic (human to human) CJD after exposure of contaminated extractive (or pituitary-derived) human growth hormone (hGH). All this concerns primary transmission. The situation at secondary transmission is unknown.

⁷ The highest recorded titres in experimental TSEs are 10^{10} i.c.ID₅₀/g.

⁸ This would imply that it cannot be excluded that these cases would appear later in the course of the epidemic.

- 5. Pathogenesis.** vCJD is considered to be due to infection with the BSE agent derived from cattle and has phenotypic characteristics and a pathogenesis which differ from other human TSEs. In humans, it is still unknown how the agent reaches the CNS and which factors (either from the host or the strain of agent, or both) may influence the spread from the periphery to the CNS. However, it is likely that it follows the same mechanisms described for the experimental TSE in animal models. In peripherally scrapie-infected mice, replication in the brain is always preceded by replication in the spleen, lymph nodes and other lymphoreticular tissues. Neuroinvasion of the PNS follows and the CNS is possibly infected through retrograde axonal transport in autonomic nerve fibres. When the infectious agent has reached the brain, it replicates at a quasi-exponential rate.

Mouse-adapted strains of scrapie and CJD produce histological lesions and PrP^{Sc} accumulation in specific brain regions characteristic for the strain. Different strains of CJD could, in humans, result in the observed clinical and pathological heterogeneity.

- 6. On species barrier.** The SSC assumes that by definition, there is a species barrier for animal to human transmission of TSE. The barrier in theory could be high or low. Current evidence suggests that it is unlikely to be absolute but it is not currently possible to quantify it. The barrier appears to be determined mainly by the strain of agent and by the difference between the *PrP* gene sequences of animal donor species and the human recipient but other host factors may also be involved. Until the advent of BSE in cattle in 1985/1986 there was no known causal relationship between human TSE of any kind and animal TSE. Thus, a critical factor that has changed this situation is the occurrence of a newly identified TSE agent, the BSE agent.

It is probable that the human *PrP* gene (*PRNP*) is of paramount importance for the determination of the size of this particular species barrier. However, the exact requirements in the PrP sequence for the species barrier are not always fully understood and may vary from one species-to-species transmission to another. The size of the species barrier for BSE-in-ruminants to BSE-in-humans is not known. In some risk assessments a barrier of the order of 1000 is assumed. However, the SSC questions these assumptions that it may be large. Until more scientific data is available, the SSC recommends that for risk assessments of human exposure to potentially BSE contaminated products, a species barrier of about 1 should be considered as a worst case scenario and that, in risk assessments, the range from 1 to 10000 is considered. The latter order of magnitude would imply that the minimal infective dose value(s) considered/accepted to be valid for intraspecies transmission of BSE animals, would also apply for humans. This may not mean that if a given dose by a given route does not produce disease in the host from which it came (usually a cow) it could not produce disease in man by the same route. This is because the longevity of man is greater and this theoretically might give time for disease to result. However there is no evidence either for or against such a notion.

- 7. On minimum infectious dose.** Calculations of infective doses are based primarily on experiments on the transmission of BSE to cattle and mice. However, if the aim of the risk assessment is to estimate the highest possible exposure, data on the transmission of all TSEs are relevant. In this case, an estimate of the highest probable titre in cattle brain is made, based on existing direct measurements in cattle which are incomplete, but also

taking into account information from other TSE infectivity measurements. This approach suggests that the dose to which humans are exposed to may be higher than existing data from BSE transmission experiments suggests.

There are several possible approaches to the calculation of the relative efficiency of different routes of infection, and hence to calculate an estimate of the effective titre of cattle tissue orally delivered to humans. Overall these various approaches yield highest estimated values from 10^1 to 10^3 ID₅₀/g cattle oral doses from a brain from a clinically affected animal. The higher value may represent a worst case scenario if the oral route is more efficient than data suggest and if a particularly high titre of infected brain is sampled. Nevertheless the possibility that such a high dose might be encountered cannot be ruled out. The SSC recommends that, in risk assessments, the whole range from 10 to 1000 ID₅₀/g cattle oral doses is considered.

- 8. Risks from exposure to amounts of infection below the minimal infectious dose.** In terms of risk assessment, the risks resulting from (low) residual infectivity should at present - until further evidence is available - be calculated as fractions of a population that would be exposed and *possibly* get infected. As it is presently not known if a threshold dose of the TSE agent exists below which there is no risk of establishing an infection and in the absence of knowledge of the shape of the dose-response curve in the low dose range, the SSC suggests using the probability scenario in the TSE context, assuming for the time being a linear dose-response curve down to the low dose range⁹. This is a conservative assumption. and would mean, as an example, that a product containing an evenly distributed residual infectivity of 10^{-3} ID₅₀/g and given to each of 1 million individuals, may result in 500 individuals being infected¹⁰.

In addition, repeated exposure could possibly increase the risk both in absolute number of cases and the likelihood that exposure would result in *effective* infection. The SSC thus suggests that the accumulative infectivity scenario is valid, provided the interval of administration is not too long (probably less than about 2-3 days) and that the repeated doses are sufficiently high so that an infective dose is reached in steady state (the repeated individual doses must be higher than the capacity of an individual to inactivate the infectivity/agent during the interval of administration).

⁹ Whether the dose/response relationship for low levels of potential residual infectivity in products after appropriate processing and handling (i.e., after appropriate sourcing, removal of SRMs, processing, avoidance of cross-contamination, etc.) is linear or follows for example a Poisson distribution, does not immediately affect the outcome of the assessment as such (in terms of absolute numbers of people at risk) but may affect the perception of the risk in management terms.(See report)

¹⁰ This is just an example; elements such as the weight of the inoculum and of the infectious material or the weight of the bovine material to which the individual was exposed **to are not taken into account.**

II. DEFINITIONS AND CONCEPTS

1. Infectious dose

An infectious dose is the dose able to establish infection. The dose is expressed as the titre (ID_{50}/g tissue in a specified species/animal strain by a specified route of inoculation - see further) x amount of tissue (g).

Infection, meaning evidence of replication of the agent, can be indirectly identified by clinical, pathological, biochemical or bioassay methods.

For humans, the infectious dose cannot be calculated directly, but must be related to animal models. The relation of the two species barriers of interest, i.e. between cattle and mouse, and between cattle and man is unknown.

2. TSE Agent Strains

In the absence of genotype information (nucleic acid sequence), strains of any infectious agent are defined by a series of stable distinguishing phenotypic properties. In TSEs they are defined after serial passage in laboratory animals and are usually distinguished by their relative incubation periods in animals of the same and of differing PrP genotype and the distribution of histopathological lesions. Electrophoresis patterns of PrP^{res} and glycoform ratios may be considered as molecular characteristics of some given strains of TSE agents.

3. PrP^{res} and PrP^{Sc}

PrP^{Sc} is a general term used for PrP that accumulates during TSEs. It is normally considered to be associated with TSE infectivity, and often used as a surrogate marker for infectivity. (See also Prusiner, 1982; Raymond *et al*, 1997 and Kocisko *et al*, 1999). However, failure to detect PrP^{Sc} does not necessarily imply absence of infectivity¹¹

PrP^{res} is an operationally defined term. It is an altered form of PrP that is partially resistant to proteinase K digestion. It does not necessarily cause infection. Production of PrP^{res} in vitro in a cellular experimental model has so far not resulted in infectivity as detected in mouse bio-assays. (See also Hill *et al*, 1999.) (It should however be noted that it is possible to produce infectious PrP^{res} in vitro, with neuroblastoma cells that are chronically infected. (Race *et al*, 1987; Butler *et al*, 1988).

The two terms are sometimes used as synonyms. However PrP^{res} was introduced to emphasise the operationally defined properties and to distinguish it from theoretical debate about the relationship between PrP and the infectious agent.

4. Titre

Titration is measurement to an endpoint. For infectious agents it is a method by which, from a series of serial dilutions, the endpoint is determined by the highest

¹¹ Apart from the conformation dependent assay, it is impossible to measure anything else but PrP^{res}.

dilution from which infectious agent can be detected. The titre is calculated from a determination of the dilution required to induce disease in 50% of animals.

In a TSE context, titre is a measure of the concentration of infectivity in a unit mass of tissue. Its purpose is to make comparisons of levels of infectivity (and thus risk) in unit masses of different tissues or, the same tissue in different regions or, the same tissue from different animals of the same species. The system of measurement would need to be identical in each case e.g. mouse i/c ID₅₀/g.

Current biochemical methods do not allow to detect PrP^{Sc} in amounts below approx. 10³ - 10⁴ infection units (or ID₅₀, see below)

5. LD₅₀ or ID₅₀?

In toxicology: The “LD₅₀” is a concept developed to test chemicals for their acute toxicity. The classical procedure for determining the acute toxicity of a new compound consists of establishing the dose response curve for lethality after single-dose administration of the compound to groups of experimental animals. The animals are observed after the administration of the compound for a minimum of 24 hours. If the animals appear to be healthy at the end of this period, they are examined at daily intervals for at least one week for appearance of delayed toxicity. The LD₅₀ is then statistically leading to death in 50% of the animals. Therefore, the LD₅₀ does not describe the shape of the dose-response curve¹² and does not necessarily relate to the shape of the dose response curve it was derived from.

In a TSE context: The ID₅₀ concept is an operational definition meaning the dose that will establish infection with disease in 50% of the challenged animals. Infection, meaning evidence of replication of the agent, can be indirectly identified by clinical, pathological or bioassay methods. There are insufficient data to know whether there is a strict dose/response relation with respect to TSE agents. This needs further research.

TSE infections in susceptible animals or breeds usually have long incubation periods, the disease is suspected only after the onset of clinical signs, is still incurable and can only be confirmed with certainty after death. Thus LD₅₀ and ID₅₀ appear to be almost equivalent in this context although in some circumstances the LD₅₀ value may be an underestimation of its real value of an infection. By using the concept “ID₅₀” this underestimation could be accounted for, not only on theoretical grounds, but also in animal experiments in which individuals are infected (and from which replicated infectivity can be recovered, hence excluding inoculum) but which do not succumb to clinical disease *i.e.* are carriers. This could occur for example in experimental scrapie in C57Bl6 mice infected with strain 22A, when the incubation period exceeds the normal mouse life expectancy (Dickinson *et al*, 1975). This phenomenon has been demonstrated in an experimental model. C57BL6 mice were

¹² This means that a dose of a compound leading to death in 5 out of 10 animals (the LD₅₀) will not necessarily lead to death in 5 out 100 animals when 1/10 of this LD₅₀ is given. Depending upon the steepness of the dose-response curve , 1/2 of the LD50 may lead to no death in animals at all or may still kill 30% of the animals.

injected intraperitoneally with the 22A strain of mouse passaged scrapie and the animals allowed to live into old age, approx 630 days. Although no animals showed clinical signs or histopathological lesions of scrapie, infectivity was found in their spleens (Dickinson et al, 1975). These authors also state that “the detailed titre levels and time sequences for the start of replication in the spleen depend on the strain and dose of the agent, on the genotype of the mouse and its age at injection.”¹³

It is suggested that, in documents of the TSE/BSE *ad hoc* Group and of the Scientific Steering Committee the term “ID₅₀” should be used. However, it is seldom practical (and has rarely been done in practice) to determine whether or not infection has occurred. What is usually determined, is the occurrence of clinical TSE, which is always fatal.

6. The use of the “ID₅₀ concept” in a TSE context.

The response to a particular agent strain depends not only on the dose, but also on the species, the route of infection and, where relevant, other factors such as the breed or strain of the host (assumed to be related to the *PrP* gene sequence of the donor, recipient species and other unknown host factors). “ID₅₀” values should therefore always be expressed as a ID₅₀ value for a given route of infection, a given species and a given breed:

Species , breed¹⁴ of the host, route, ID₅₀ per unit of mass
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7. The sensitivity of the mouse assay relative to the cattle assay for measuring infectivity derived from cattle brain is at least 1000-fold less sensitive.

8. Efficacy of transfer: Efficiency of TSE infection depends on the strain of TSE agent, donor and recipient host factors and the route of infection. It is not possible to extrapolate the relative efficiency of i/c to oral route from other infectious diseases. Primary transmission may be more efficient (and/or result in a shorter incubation period) by the i/p route than by i/c route.

It might be possible to select variants with given biologic properties using one given route of inoculation : for example, infection by oral route may result in the selection of agents that have greater ability to infect recipient by oral route than those adapted to their new host by i/c route (Carp et al, 1997).

The efficacy of transfer under natural conditions, is not known.

9. Species barrier. This is the natural resistance to transmission when documented one species is exposed to the TSE of another.

¹³ One should, however, keep in mind that strain 87V, inoculated i/p to IM mice, never results in disease although the agent is present in the spleen and even replicates in the spleen to high titres. In this case there is infection without lethality but CNS neuro-invasion does not occur (Collis and Kimberlin, 1985).

¹⁴ "Strain" for laboratory animals.

III. SOURCE FACTORS

III.1. INTRODUCTION

There are two main variables that need to be taken into account when attempting to determine the magnitude of the human infective dose of the BSE agent. These are the strain of agent and the PrP genotype of the host species.

However, in regard to the host species there is a sub-set of variables to consider. These variables include the phylogeny, breed and *PrP* gene sequence of the host species, the source tissue, the infectivity titres within tissues, the conformation and possibly the glycosylation pattern of the PrP^{Sc}. Each of these factors will be discussed below in an attempt to identify those that may significantly influence the development of infection and disease when a certain dose of agent has been consumed (eaten) by man, applied (*e.g.* cosmetics) or administered (*e.g.* medicinal products).

It is also important to remember that there could be an occupational risk (*e.g.* for abattoir workers, veterinary pathologists, zoo workers and knacker men). There is a possibility of exposure in abattoirs from healthy but infected animals and tissues from them. In addition there may be an occupational risk from contact with infected tissues from species that are neither consumed nor used as sources for the preparation of cosmetic or medicinal products. Any risks there may be for such workers are more likely to involve only parenteral challenges as very few of these species are eaten. The route of exposure is dealt with in another section of this report. Occupational risks may in certain cases also exist even if there is no tissue infection. For example, infectivity could reside in feed still in the lumen of the digestive tract if the animals were fed with contaminated feed before slaughter or death.

Of the animal TSE agents known there are at least four groups: scrapie and BSE that are well defined, TME that exists as at least two strains (Bessen and Marsh, 1994) and chronic wasting disease (CWD) about which little is known. TME affects only mink which is not a food animal species and will not be considered further. CWD affects various species of *Cervidae* but only in North America, a region devoid of reported cases of BSE in native-born cattle. Some cases of sporadic CJD have occurred in deer hunters in the USA but the origin of disease is not known.

The group is asked to focus on exposure to the BSE agent rather than TSE agents in general. These TSE include scrapie in sheep that may have been the origin of BSE. However, there is no evidence that sheep, or goats, infected or affected with scrapie agents have historically been a danger to man. Thus this section will focus mainly on host species that have succumbed in the field to TSE in the BSE era.

III.2 VARIABLES IN THE AGENT

III.2.1. The strain of agent

The agent that causes BSE in cattle appears to exist as a single, major, stable, biological strain, as determined by the incubation period and lesion profile in in-bred strains of mice, no matter what is the geographical source: for the UK see Bruce (1996) and for Switzerland see Bruce (1994) and R Fatzer (personal communication). The possibility of currently existing or newly developing strains isolated from cattle cannot be ruled out. This is certainly possible, given the evidence for strain diversity in CJD

(Bruce *et al* 1997; Telling *et al*, 1996; Collinge *et al*; 1996; Parchi *et al* 1999), TME (Bessen and Marsh, 1994) and in sheep scrapie (Bruce, 1996) and the fact that mutant strains can be produced and selected by other species than those in which they arise (Kimberlin *et al*, 1987; Kimberlin, Walker, *et al*, 1989). Strains can also mutate in the same species (Bruce and Dickinson, 1987).

The biological strain type of the BSE agent is different from strains of scrapie agent or of the agents isolated from cases of sporadic CJD. Of particular note is the fact that the strain of agent isolated from cases of vCJD has closely similar biological properties to the BSE agent derived from cattle (Bruce *et al*, 1997; Scott *et al*, 1999)¹⁵. Furthermore the molecular properties of agent determined by analysis of the fragment sizes and glycoform patterns in Western blots of PrP^{Sc} isolated from humans with vCJD and mice inoculated with either vCJD or BSE are also closely similar (Hill *et al*, 1997).

Molecular strain typing (Hill *et al*, 1998,) of PrP^{Sc} from natural cases of scrapie and a limited amount of biological strain typing of isolates from British sheep (Bruce, 1996) has provided no evidence yet that BSE in these species is present.

Interestingly a historical isolate from natural scrapie in British sheep (CH1641) had a very similar molecular profile to that of BSE agent but could be readily distinguished from it by its transmission characteristics in mice (Hope *et al*, 1999).

III.2.2 Significance of the hypotheses of TSE agent structure

A range of species has succumbed in the field to newly described TSE infections during the BSE era (Annex 1). The question now arises as to whether the agents that cause BSE and BSE-related TSE (such as natural FSE in domestic cats, captive wild species and experimental BSE in sheep and goats) are identical, or merely show identical biological and molecular properties. This depends upon whether TSE agents are entirely comprised of proteins, *i.e.* are prions, or if they contain a conventional genome presumably of a small nucleic acid *i.e.* are virinos or unconventional viruses (Schreuder, 1994). If the former hypothesis is true, the agents are clearly not identical since the PrP forming them varies depending upon the molecular sequence of nucleotides in the *PrP* gene in the coding region and thus, potentially or actually, the amino acid sequence of PrP. The biological and molecular strain of agent isolated from cattle with BSE and humans with vCJD could still be the same if the PrP was folded in similar ways *i.e.* the conformation was similar (Safar *et al*, 1998). Alternatively the virino and unconventional virus hypotheses could explain strain variation by conventional concepts. In this situation the agent genome could be identical in the agent strains isolated from cattle with BSE, humans with vCJD and other BSE-related diseases, whilst the associated PrP was chemically different, even if misfolded in a similar way.

If the agent is a virino, isolates from any species could contain the same agent genome as is present in isolates from cattle with BSE. If the incoming agent is an infectious

¹⁵ Bruce *et al* (1997) studied transmission of vCJD into wild type mice in order to identify the biological properties of the agent. Scott *et al* (1999) investigated the transmission of vCJD and BSE into mice transgenic for the bovine *prnp*. Their results supported the Bruce *et al* results with respect to the human to cattle route.

protein, or prion, the conformational structure appears to be critical if disease is to result. Any new risk to man from BSE derived from sheep for example would therefore have to be due to one of two possibilities. Either the sheep contained the BSE agent genome (virino or virus hypotheses) or the PrP^{Sc} had a conformation of a type hitherto unknown (prion hypothesis). This is equivalent to saying that the extent of the species barrier is determined by the strain of agent (virino or virus hypotheses assumed) and by the variation in the *PrP* gene sequence between the donor species and the recipient species (SEAC, 1994).

Whether 'BSE' agent isolated from a sheep or goat would behave in precisely the same way in humans as it does in in-bred mice will depend partly on what is the precise agent structure.

In natural sheep scrapie a number of strains exist. Sometimes more than one strain of agent can be isolated from a single sheep. If scrapie exists in sheep in a country and there is also a risk from BSE, it is theoretically possible that dual (scrapie and BSE) infections could occur. Furthermore if new strains of BSE develop in the future (or already exist even though currently unknown) dual infections of BSE could arise. The consequences for the recipient host from such potential or actual natural exposures are unknown. In this report it will be assumed that if BSE and scrapie infection coexist then there is the risk from the BSE agent and this risk will be no different than that from a pure BSE infection.

III.3. VARIABLES IN THE DONOR

III.3.1. Phylogenic considerations (and host range)

Krakauer *et al* (1996) reconstructed the phylogeny for various mammalian species based on the *PrP* gene sequence. They identified two pairs of derived substitutions uniquely shared by cattle and the hominids. They suggest that a consequence of the selection process might have predisposed humans to prion disease arising in cattle. However, neither the phylogenic classification of a species nor knowledge of the *PrP* gene sequence of a species permits a prediction to be made of the cross species transmissibility of a spongiform encephalopathy. For this to be feasible a much better understanding of the molecular basis of the strains of these agent is required (Goldmann *et al*, 1996).

A number of studies have been made of the *PrP* gene sequence in different species affected by BSE-related TSE (*e.g.* Poidinger *et al*, 1993). A substantial study of 27 mammalian and 9 avian PrPs has been reported by Wopfner *et al* (1999). None of these analyses has identified a common thread that enables a prediction to be made that a particular species is susceptible to a TSE or conversely is resistant. Only artificially produced PrP knockout (null) mice have the latter distinction. Even there the dogma has not been challenged with all known strains of agent.

Some species (rabbits for example) are refractory to challenge with prions of any kind so far used. Other species such as hamsters and chickens are resistant to challenge with some agents (BSE for example), even via the i/c route (Annex 1). An alternative view is that all species have their own sporadic TSE that for the most part are undiscovered (CJ Gibbs *jr*, personal communication).

Sheep scrapie, a possible origin of BSE has been transmitted to a wide range of experimental hosts but it occurs only in three species naturally – sheep, goats and moufflon that are closely phylogenetically related. New TSE in animals in the field in the BSE era have occurred in over fifteen species but these all belong to the families *Bovidae*, *Felidae* and possibly primates, though several other families of animals have been exposed.

Host range

Field disease

A range of species have naturally succumbed to newly described TSE infections during the BSE era (Annex 1). Agents with the biological characteristics of the BSE agent from cattle have been isolated from a nyala, a greater kudu and domestic cats. It is presumed, but not proven, that all the species that developed natural TSE in Annex I have been infected with the BSE agent following consumption of infected CNS tissue derived from cattle (captive wild carnivores) or *via* MBM (captive wild herbivores). Of these animals with naturally-acquired disease, only cattle are likely to be consumed by man or provide tissues or products for use in cosmetics or medicinal products and devices if current EC rules are enforced.

It is entirely possible that a wider range of natural orders of species has become infected with scrapie or BSE than has succumbed to disease. Such a situation has been suggested to occur in British zoos (Kirkwood and Cunningham, 1994). Such unidentified species could succumb after long incubation periods. They are only likely to be identified if clinical disease results at a significant incidence and there is effective surveillance. If no disease results, such infections, if they exist at all, may remain undiscovered.

Experimental host range

A further range of species has been experimentally challenged with BSE by either the i/c, or oral routes, or both and those that have succumbed are also listed in Annex 1. Of these species only sheep, goats and pigs are consumed by man or provide products that are used in cosmetics, or medicinal products, or devices. Pigs did not succumb to infection when large amounts of brain from clinically BSE-affected cattle were administered by the oral route. Pigs are susceptible to BSE but only by multiple parenteral routes with a high dose of untreated brain material. However, the small numbers of inoculated animals on one hand and the lack of knowledge of the effect of the genetics of PrP in pig with respect to its susceptibility to TSE agents on the other hand, should be kept in mind. Sheep and goats did succumb to experimental oral infection (Foster *et al*, 1993). It is likely that some sheep were naturally exposed to BSE-infected MBM though to date there is no evidence that natural BSE has ever occurred in sheep or goats anywhere in the world. Nevertheless the difficulty of distinguishing scrapie from BSE in sheep and goats at the clinical and pathological

level is a matter of great concern. Chickens are not susceptible by either the oral or parenteral routes (Bradley, 1996)¹⁶.

What is clear from the results of the experimental challenges of farmed animals with the BSE agent is that cattle, sheep and goats are susceptible to BSE by the oral route. Of the susceptible species only cattle have succumbed as field cases of disease. It is therefore this species that presents the highest potential or actual risk to humans because cattle products are widely eaten and/or used as ingredients or in the manufacture of medicines, medicinal devices and cosmetics. There is also a potential concern about sheep and goat products that are also widely eaten. Cosmetics and some medicines or devices may contain sheep origin materials as ingredients or they may be used during manufacture.

It is noted that iatrogenic scrapie has occurred at least twice in the last 70 years following the use of vaccines prepared in part from sheep brain.

III.3.2 Breed considerations

In cattle within dairy breeds in the UK, at least, there seems to be no difference in susceptibility to BSE. The numbers of cases of BSE within dairy breeds is directly related to the numerical size of the breed (Bradley and Wilesmith, 1994)

In sheep, susceptibility and resistance to natural and experimental scrapie appears to be controlled by the *PrP* gene either operating *via* its influence on the incubation period or affecting susceptibility.¹⁷ The influence of the *PrP* gene on the response of sheep to exposure to scrapie is discussed in the next section.

There is as yet no evidence for a difference in susceptibility between goat breeds. Goats appear to be uniformly susceptible to scrapie. Nevertheless, in goats, the polymorphism of the *PrP* gene of codon 142 governs the duration of the incubation period in regard to experimental challenge with the BSE agent, scrapie strain CH 1641 and sheep passaged ME7 scrapie strain.

III.3.3 *PrP* gene sequence considerations

Cattle

Goldmann *et al* (1991) reported that different forms of the bovine *PrP* gene have five or six copies of a short, G-C-rich element within the protein-coding exon. Subsequently Hunter *et al* (1994) examined the frequency of these octapeptide repeat polymorphisms in healthy cattle and cattle with BSE in Scotland. There were no differences between the frequencies of these *PrP* genotypes in healthy cattle and cattle with BSE. The structure of the *PrP* gene of Belgian cattle (Grobet *et al*, 1994) and US cattle (McKenzie *et al*, 1992) seems no different from that of British cattle. These studies collectively show that the 6:6 repeat is the most frequent, the 6:5 is moderately frequent and the 5:5 repeat is rare. The *PrP* gene of cattle does not seem to exert a

¹⁶ The SSC opinion of 17 September 1999 on the *Risk born by recycling animal by-products as feed with regard to propagating TSE in non-ruminant farmed animals.*, points at the possibility that infectivity may be present in the digestive tract of poultry in the hours and days following feeding with contaminated products.

detectable influence on the occurrence or incubation period of BSE. Hau and Curnow (1996) studied the incidence of BSE in related animals and developed a statistical model. They concluded that there was still no evidence, molecular or statistical, for genetic variation in susceptibility. There is also no report that PrP^{Sc} resulting from cattle with different *PrP* gene sequences is more or less able to establish infection or disease in other cattle or in other species.

Sheep

Within sheep there is a considerable variation in the sequence of *PrP* genes. This variation is summarised by Dawson *et al* (1998). In sheep, susceptibility and resistance to natural and experimental scrapie appears to be controlled by the *PrP* gene either operating *via* its influence on the incubation period or affecting susceptibility. Thus, if the incubation period is lengthened beyond the natural lifespan of exposed sheep, they would not exhibit clinical disease. At present it is not known if such sheep are carriers of infectivity, though experiments to determine this are in progress. The knowledge gained from *PrP* genotyping, clinical, pathological and epidemiological data in sheep in flocks with and without scrapie has led to the use of genetic selection of 'resistant' sheep¹⁷. The objective is to increase the incidence of 'resistance' alleles and reduce the incidence of susceptibility alleles. There is considerable evidence from the field that sheep carrying the 'resistance' survive challenge with natural scrapie and that the incidence of scrapie in a previously high incidence flock declines. Other than a small number of challenges of Cheviot sheep (from a flock in which natural scrapie also occurred (Foster, Fraser and Hope, 1993, Foster *et al*, 1996), there is little information on how selected resistant sheep would respond to BSE challenge under natural conditions. However, further oral challenge experiments in Cheviot and Romney Marsh sheep are in progress. Nevertheless it is abundantly clear that the *PrP* genotype of sheep determines the risk factor for sheep. A range of possible *PrP* genotypes can succumb to scrapie but it is not known what risk these different genotypes (if infected) pose to another species and whether there is no difference, a small difference, or a large difference in scrapie transmission risk from them.

Goats

Goldmann *et al* (1996) analysed the *PrP* gene of goats of mixed breeding and found several different alleles. Four PrP protein variants were inferred, three of which appeared specific to goats and the fourth was shared commonly with sheep. The dimorphism at codon 142 (isoleucine / methionine) resulted in an increase in incubation period following experimental challenge with BSE agent, CH1641 and sheep-passaged ME7 scrapie agents.

Subsequently Goldmann *et al* (1998) reported the identification of the shortest known *PrP* gene of any species in goats. This has only three octapeptide repeats in contrast to five or six that commonly occur in several other species including sheep, cattle and man. In humans there is a wider variation of octapeptide repeat numbers. Insertions of

¹⁷ For a more extended viewpoint on 'scrapie resistance' see: Scientific Opinion adopted by the SSC on 22/23 July 1999 on *The policy of breeding and genotyping sheep, i.e. the issue of whether sheep should be bred to be resistant to scrapie.*

additional repeats are usually associated with TSE occurrence in humans. Fifteen out of 111 goats carried the three octapeptide repeat allele. One heterozygous goat (3:5 repeats) and four homozygous, 'normal' (5:5 repeats) were experimentally challenged with SSBP/1 by the intracerebral route. The incubation periods for the latter four goats were 620 (\pm 23 SEM) days whereas the former had an incubation period of 968 days. These studies show that there is variation in the caprine *PrP* gene and that at least some alleles are associated with lengthened incubation period following experimental challenge with TSE agents. There is currently no evidence that any of the alleles so far identified are pathogenic, *i.e.* cause disease in the absence of infection. Neither is it known if any of the various PrP structures from goats assist or restrict the transmission of TSE from an infected goat to another species.

III.3.4 Tissue considerations

Robinson *et al* (1990) using the 263K strain of scrapie inoculated into the brain of hamsters showed that the slope of the dose response curves, or average rate of change *per* dilution, was different for the brain and spleen from the challenged animals. The authors considered that the homologies of the pathogens in the different organs or the pathogenic mechanisms become ambiguous. They claimed that this ambiguity invalidates the assumption that the infectivity titres of compared inocula are measures of the same pathogenic phenomenon.

Titres in different tissues and different periods of incubation or clinical disease:

Infectivity titres in the brains of different sheep are variable even in the clinical phase of disease (Hadlow *et al*, 1979; 1982). There is some evidence to suggest that this may be breed dependent (Hadlow *et al*, 1979). But it could also be due to the different sensitivities of mice to different agent strains or to variation in PrP from sheep of probably different PrP genotypes.

Different parts of the CNS of the same sheep may show a variation of titres with significant differences between them (Hadlow *et al*, 1982). Comparable studies in cattle have not been reported but variation in extent of pathology and abundance of PrP^{Sc} (Hope *et al*, 1992) and scrapie associated fibrils (SAF) in different CNS regions suggests that a similar situation also exists in cattle or in other species affected by the BSE agent.

Titres in lymphoreticular system (LRS) tissues in natural scrapie in Suffolk sheep may be significant once the zero phase (*i.e.*, the period post-infection in which infectivity cannot be detected in the host or in a particular organ) is complete, with the CNS only becoming detectably infected at about 25 months of age and before the onset of clinical signs. At this time the titres in the CNS are lower than in the LRS. In the clinical phase of disease titres in the CNS usually exceed those in the LRS.

The pathogenesis, and hence the tissue distribution of infectivity in cattle with BSE and sheep with scrapie is different (Foster *et al*, 1996).

III.3.5 Titre considerations

In regard to titres, it is generally accepted that the highest titres of infectivity are found in the CNS (brain and spinal cord) of TSE-affected animals in the terminal phase of the clinical disease. Infectivity titres in non-neural tissues at this time are generally lower than those in the brain. The same may apply at the point of onset of clinical signs (*i.e.* end of the incubation period), or for a restricted period before. However, following a short zero phase after peripheral exposure and until later in the incubation period the infectivity titre in non-neural tissues that are infected at detectable levels in mice, is likely to exceed CNS at the same time point. Since TSE disease results from a dynamic process the actual titres will vary during the course of the incubation period.

A series of BSE-infected cow brain samples have been tested for the presence of BSE infectivity in mice (Fraser *et al*, 1992; Taylor *et al*, 1994; Taylor *et al*, 1995; Schreuder *et al*, 1998). From these data, values for the ID₅₀ in the donor cow brain samples (all from clinically affected animals) can be estimated. The values range from approx. 10³ to 10⁵ per gram of cow brain, as measured by the intracerebral route in mice. Highest values were obtained from samples with a high proportion of brain stem in the sample, lower values were obtained when brain stem was omitted. The other possible source of variation is the degree to which the clinical disease had progressed before slaughter. Animals slaughtered early in the clinical phase possibly may have lower levels of infectivity than those which have progressed further into the clinical disease. There is evidence for an increase in infectivity throughout the clinical phase in some experimental models and a plateau of infectivity in other models. Results from an ongoing BSE pathogenesis experiment (Wells *et al*, 1998; Wells *et al*, 1999) could clarify this question.

Highest TSE titres

It should be noted that in certain experimental models, e.g. the 263K scrapie strain in hamsters and the 301V murine mouse adapted BSE strain in mice, titres in the CNS can reach 10¹⁰ ID₅₀ per g. This value perhaps represents the highest possible value for any TSE.

Data from a Veterinary Laboratories Agency (VLA) experiment (unpublished but in the public domain) suggests that the measurement of BSE infectivity in mice is about 10³ fold less efficient than in cattle when the i/c route is used. In this experiment BSE i/c titrated in cattle gave a value of at least 10⁶ per gram (experiment still ongoing) and in mice a value of 10³ ID₅₀ per g (experiment concluded). Given that mouse i/c titres up to 10⁵ have been obtained, this suggests that titres of up to 10⁸ cattle i/c ID₅₀/g may be found at least in some parts of the brain from animals with disease. (This estimate was obtained for animals in the latest stages of disease. It does not preclude similar high titres in earlier stages).

In field cases of BSE in cattle tested so far and thought to be caused by MBM, infectivity has not been recorded outside the CNS (brain, spinal cord and eye) in mouse tests. In experimental orally-induced BSE infectivity has been found in the distal ileum at intervals during the incubation period starting six months after exposure (Wells *et al*, 1998). Furthermore, central nervous tissues and dorsal root and trigeminal ganglia were found to be infective shortly before the onset of clinical signs. In addition, at one point

only in the clinical phase of disease, sternal bone marrow was also found to be infective though one of the possible explanations given by Wells *et al* (1999) is that this could have resulted from cross contamination. Nevertheless inconsistent and low titre genuine infectivity cannot be ruled out.

In three goats with natural scrapie, Hadlow *et al* (1980) found little difference in titres in the brain. However in non-Suffolk sheep maximal titres could be lower (10^3 to 10^4 mouse i/c ID₅₀ per 30 mg of brain lower) than they were in Suffolk sheep though the titres varied in different parts of the brain (Hadlow *et al* 1979). (The maximum titres in the brains of 5 Suffolks with scrapie were 4.0, 4.3, 4.4, 6.1 and 6.5 logs. In 5 sheep of other breeds they were 1.4, 4.7, 4.4, 1.4 and 4.3 logs /30 mg of brain.)

During the incubation period of natural scrapie in Suffolk sheep infectivity titres were absent in the early stages but present at low titre from about 25 months of age being in the region of $10^{0.7}$ to $10^{2.3}$ mouse i/c ID₅₀ per 30mg of brain, *i.e.* some 3 to 4 logs lower than in the clinical phase of disease.

In natural goat and Suffolk sheep scrapie respectively, Hadlow *et al* (1980, 1982) found a range of titres in different parts of the brain in the same animal that could amount to about 10^3 mouse i/c ID₅₀ per 30mg of tissue. Such studies have not been reported in cattle with natural or experimental BSE, or in sheep with experimental BSE. However, study of the amounts of PrP^{Sc} and SAF in different parts of the brain of cattle with natural BSE were variable (Hope *et al*, 1988) with the basal ganglia > medulla > spinal cord > cerebellum and cortex. This accords with the distribution of microscopic lesions in the brain and spinal cord.

Assay of experimental BSE infectivity in sheep and goat brain and spleen during the clinical phase of disease has been reported by Foster *et al* (1996). Based on the incubation period lengths in mice inoculated i/c, there was significant infectivity in the spleens of these animals.

When measuring titre it is important to note that titres will be higher if measured with species by the i/c route. They are likely to be lower when measured in a different species and account should be taken of the fact that the i/c route may not produce the shortest incubation period.

The relationship between the amount of PrP^{Sc} and infectivity titre is depending upon the strain and the host; for example, in hamsters, two TME strains can be identified, Hyper and Drowsy. These two strains are differentiable by their clinical signs and by the amount of PrP^{Sc} that accumulates in vivo in infected individuals. In humans, FFI patients are known to have low amounts of PrP^{Sc}. Moreover, in the C57Bl6 syngenic mice, inoculated with mouse-adapted BSE or mouse-adapted scrapie, the infectivity titres are in the same range (10^9 ID₅₀/g). However, the amount of PrP accumulated is significantly greater for the scrapie than for the BSE strain. (Lasmézas, 1996). Recently, Safar *et al* (1998) published a Conformation Dependent Assay that can evidence differences in PrP conformation; their experimental data strongly support the existence of abnormal PrP that does not resist proteinase K digestion; this result could explain the inter-species transmission of TSE without PrP^{Sc} accumulation (Lasmézas *et al*, 1997).

III.3.6 PrP^{Sc} structural considerations

Priola (1999) reviews the evidence supporting the view that similarities in the primary structure of PrP in the donor and recipient species, especially in particular domains, aids the transmission of TSE across species boundaries. Conversely, differences in the primary structure in the middle third of the PrP molecules may significantly obstruct the process of PrP^{Sc} formation in the recipient species. (For further discussion see Section VI below on species barriers.)

Assuming that TSE agents are infectious proteins, the molecular basis for strain variation is believed to be encoded by the conformation of PrP^{Sc} (Prusiner, 1982; Safar *et al*, 1998). Since the conformation of PrP^{Sc} derived from most natural cases of TSE is not known it is not possible to predict whether or not the protein from any particular species presents a risk. For example, over some 250 years scrapie from sheep has not been regarded as a human pathogen because there is no epidemiological support of an origin in sheep of any form of CJD in man (SEAC, 1994). Thus if BSE agent did occur in sheep and the agent is a prion, a new conformational structure not previously experienced could explain pathogenicity for man if it were to occur. Alternatively, exposure of man to PrP derived from a *PrP* genotype from sheep resistant to scrapie but susceptible to BSE could explain pathogenicity for man as such a situation would not have occurred before the BSE era.

Thus the different glycosylation patterns and molecular strain types of PrP could theoretically explain different pathogenicities for different species including man. There is evidence that the molecular strain type of agent can differ in different organs of the same animal affected with scrapie. The possibility that organ-specific modification of infectivity is due to modification of the infectious units is supported by the fact that PrP^{Sc} from different tissues (spleen and brain of mice infected with 139A scrapie strain, (Rubenstein *et al*, 1991) and parts of the brain, (Somerville and Ritchie, 1989) may be glycosylated to different degrees with a variation in the ratios of diglycosylated : monoglycosylated : unglycosylated PrP (Somerville, 1999). The glycosylation of PrP^{Sc} depends¹⁸ on the strain of agent, the region of brain and on the PrP genotype.

III.4 SUMMARY ON SOURCE

Of animal TSE agents, BSE is at present the most important human pathogen. Other animal pathogens cannot be ruled out. Cattle are by far the highest potential risk species for transmission of BSE to man. Risks cannot be excluded for sheep and goats although no indication of such risk exists today. (See also the SSC opinion on BSE in sheep, adopted in September 1998). Animals in the advanced clinical stage of disease present the highest risk. Lower risks occur earlier in the clinical phase of disease and in the immediate pre-clinical phase. The CNS is the highest risk tissue because it can develop the highest titres. LRS tissues present a lower risk but can be infected from an early stage after exposure.

¹⁸ It should be noted that PrP^{Sc} has never been isolated. All research work is presently being done on PrP^{res}.

The critical factors that determine whether or not infection with the BSE agent would cause disease in an exposed human depend upon the strain of the agent, the infectivity titre of the agent, the efficiency of the route of infection, the interaction of the agent with the host (and possibly the PrP genotype of the host). Thus at the point where a human is exposed to the BSE agent even if all the factors are known, the one of most significance is likely to be the dose but even then no predictions can be made of the outcome as there are major uncertainties about the infectiveness of animal-derived TSE agents for humans.

IV. HOST FACTORS

IV.1. Genotype

According to present knowledge, susceptibility to human TSEs resides at least partly in the *PrP* gene, *PRNP*. Other unknown factors may contribute to TSE susceptibility and/or incubation period. For example one *PrP* genotype of Suffolk sheep succumbs to natural scrapie whereas the same genotype of Cheviot sheep does not. When RIII and C57BL mice, which have the same *PrP* genotype, are injected with BSE their incubation periods differ by 100 days.

In most inherited human TSEs, it is generally assumed that the genetic aberrations alone are sufficient to cause disease. However, they may not be the only factors contributing to the appearance of the disease. In fact, the findings that patients of the same family (Masters *et al*, 1981, Collinge *et al*, 1992; Poulter *et al*, 1992; Hainfellner *et al*, 1995; Chapman *et al*, 1993; Barbanti *et al*, 1996), hence bearing the same mutation on the *PRNP* gene, show a great clinical variability, and that the penetrance of E200K mutation varies in different geographical cluster areas (Goldfarb *et al*, 1991; Chapman *et al*, 1994; Spudich *et al*, 1995; D'Alessandro *et al*, 1998), argue against the genetic only hypothesis. Moreover, recent work with mice transgenic for the P102L mutation (which associates with human Gerstmann-Sträussler-Scheinker disease, GSS) further suggests that this mutation might only be an important susceptibility factor rather than a direct cause of GSS (Manson *et al*, 1999).

The methionine-valine polymorphism at *PRNP* codon 129 seems to be important for the variability in the phenotypic expression of sporadic and inherited human TSEs (Parchi *et al*, 1999). Moreover, the polymorphic codon 129 is also important for determining the susceptibility to the disease: homozygosity for met/met is present in about 40% of the normal European population, but is found twice as frequently in sporadic (Salvatore *et al*, 1994; Deslys *et al*, 1994; Palmer *et al*, 1991) and iatrogenic (Collinge *et al*, 1991; Deslys *et al*, 1994; Brown *et al*, 1994) CJD. In a series of sporadic CJD cases, an excess of the homozygous 129 Met/Met genotype corresponded to a 3.4-fold increased risk of developing CJD (Laplanche *et al*, 1994). In contrast, heterozygosity appears to act on incubation period duration and/or to protect against CJD (Deslys *et al*, 1998). All vCJD patients tested to date have the MM129 genotype (Collinge, 1999), arguing that methionine homozygous individuals are most susceptible to develop the disease. Thus, similarly to what has occurred in iatrogenic CJD after exposure of contaminated hGH, it is possible that also val/val or heterozygous subjects will be of similar or lower susceptibility and will develop vCJD, but with a longer incubation period, i.e., later in the course of the epidemic. In Japan,

allelic frequencies and genotype distribution of codon 129 (Doh-ura *et al*, 1991) and codon 219, another polymorphic site of the *PRNP* gene (Furukawa *et al*, 1995), are significantly different from European and North American populations (Petraroli and Pocchiari, 1996). Therefore different populations may show different responses to exposure to the BSE agent.

IV.2. Age

Outram *et al* (1973) showed that neonatal mice inoculated i/p with three different strains of scrapie had much longer incubation periods than did weanling mice of the same strain. No survivors ever occurred when weanlings were inoculated with similar doses. A few neonates had shorter incubations however. Some of the neonates showed neither clinical signs nor neuropathological lesions. The mean incubation for weanlings was exceeded by 10 – 50 SD (depending on agent/host strain combinations) in the neonate survivors. This suggests that the age at exposure can have a marked effect on pathogenesis/susceptibility. The authors suggested that there could be a lack of receptor sites in neonates or the mechanism that could remove infectivity exceeded the capacity of the mouse to replicate the agent. The authors also suggested a difference between neonatal mice and sheep in that the latter had much better developed immune systems at birth and this might account for the observed maternal transmission of sheep scrapie but its absence in mice.

Dickinson and Taylor (1988) reported that a major peculiarity of scrapie is that for an animal to become infected by a ‘natural’ route (i.e. not directly into the CNS), it must be immunologically mature. A major difference between mice (or mink, human, pig etc.) and sheep (or goats deer, cattle and horses) is that only the latter group become immunologically competent as embryos. The authors indicated that it would not be surprising if a similar condition eventually was found in horses.

The age at exposure therefore may play a role in the ability of the infective agent to establish itself and replicate at least in some species that are not fully immunologically competent at birth. Because the immunological system of humans is not mature at birth it could be that there is an age-based resistance lasting for an indeterminate period until it is mature. The information is meagre on this point but nevertheless, for public health concerns, all ages of humans should be equally protected from exposure.

In sporadic CJD, most of the cases occur in the 7th decade (mean age at onset in the UK 65 years, Ironside, 1999) and the disease is rare below the age of 40 (Brown *et al*, 1994; Hainfellner *et al*, 1996; Cousens *et al*, 1997; Will *et al*, 1998; Arpino *et al*, 1999; Pals *et al*, 1999). However, a small number of patients as young as 16 have been affected. Sporadic CJD can also affect people over 80.

Kuru affects adults, teenagers and very young people. The youngest kuru patient ever reported was 4 years old (Cervenakova *et al*, 1998). The age of clinical onset is presumed to relate to the age at exposure to human tissues. This can be very young including whilst still being breast fed.

Similarly to kuru and in contrast to the majority of sporadic CJD patients, the age range of vCJD patients is from 15-55 years of age at death (mean 29, data from 50

confirmed and 2 probable cases; Will et al, 1999: R.Will, personal communication). The reason for this age-distribution in vCJD is at present unknown.

In iatrogenic disease, in kuru and in vCJD, the age of clinical manifestation depends upon the incubation time. The incubation time depends upon the host's susceptibility (see IV.1. above), the infectious dose, which has not been determined, and the route of exposure. The more "central" is the inoculation site, the shorter is the incubation period (Budka, 1998): with contaminated neurosurgical instruments, stereotaxic EEG electrodes and corneal grafts, the mean incubation times for iatrogenic CJD in man were 20, 18, and 17 months, respectively (range 15-20). With contaminated dura mater grafting, it was 5,5 years (range 1,5-12). After "peripheral" inoculation through the subcutaneous or intramuscular injection of pituitary derived CJD-contaminated hormone preparations, in contrast, it was 12 years (range 5-30); the youngest victim after this application was 10 years old at onset of disease (Billette de Villemeur *et al*, 1992).

The vCJD cases observed so far are most likely to be "first generation transmission cases", not secondary infection, i.e. man-to-man after a preceding transmission from cattle to man; secondary passages may result in a shorter incubation time.

It is speculative at present whether and how much the "strain" of the agent in human TSEs is influenced by host factors and plays a role in the manifestation characteristics of human diseases, such as incubation time and clinicopathological phenotype, as is common with animal TSE strains. However, when human "strains" are molecularly defined by PRNP genotype and fragment size of PrP^{Sc} deposited in the diseased CNS, at least 6 distinct types of sporadic CJD are distinguishable which differ in age at manifestation (Parchi *et al*, 1999). When vCJD is considered, a human disease due to infection with the BSE agent which seems to behave as a single strain, phenotypic characteristics and pathogenesis differ from other human TSEs, especially with respect to prominent involvement of the lymphoid system (Collinge, 1999).

As a general rule, familial TSEs tend to manifest earlier, and to have a longer duration, than sporadic CJD. However, age at onset might vary even within the same family and codon 129 genotype (Hainfellner *et al*, 1995; Barbanti *et al*, 1996). -Usually, the PRNP codon 129 has a role in determining the age of onset and the duration of disease in both sporadic, iatrogenic and familial CJD, GSS, FFI, and kuru. In age groups of sporadic CJD below the age of 50, an excess of 129VV has been recently reported (Alperovitch *et al*, 1999). However, young cases may occur in each genotype (the youngest case in MM was 28; in MV was 20, and in VV was 23) (Alperovitch *et al*, 1999). In kuru, 129MM is over-represented in young patients, whereas heterozygous patients are over-represented in older age groups (Cervenakova *et al*, 1998). Moreover, age at the onset of the disease is associated with the duration of the disease in hGH-related CJD (D. Dormont and J.P. Brendel personal communication, December 1999).

IV.3. Transmission

Man-to man transmission of human TSEs has occurred either through medical or surgical procedures (iatrogenic CJD) or through exposure to contaminated tissues during ritual endocannibalism practice (kuru) among the Fore-speaking tribes of New Guinea.

In iatrogenic CJD, transmission of the infectious agent has always included parental exposure: use of contaminated neurosurgical instruments or stereotactic EEG electrodes, tissue (cornea, dura mater) grafting from CJD-infected donors, and subcutaneous or intramuscular injections of CJD-contaminated pituitary hormone preparations. In Kuru, the most likely entry of the infectious agent has been the oral route, although a trans-dermal or trans-mucosal route (by rubbing of bodily surfaces in children with contaminated tissues, especially brain) can not be dismissed. vCJD cases are likely to be the first example of animal-to-man transmission of a TSE agent. Although the route of entry of the infectious agent in vCJD has not yet been firmly established, it is generally believed that these patients have been orally exposed to BSE. However, since vCJD is characterised by prominent deposition of PrP^{tes} in lymphoid tissues (Hill *et al*, 1999; Hill *et al*, 1997) even before the appearance of symptoms (Hilton *et al*, 1998), demonstrating a potentially different pathogenesis from other human TSEs (Hainfellner and Budka, 1999; Kawashima *et al*, 1997), there are concerns about a potential man-to-man transmission of vCJD through contamination of blood, blood products and tissue grafts derived from persons harbouring vCJD infectivity.

In humans the mechanisms through which the infectious agent spreads from the periphery to the CNS is unknown. However, it is likely that it follows the same mechanisms described for the experimental TSE animal models. In peripherally scrapie-infected mice, replication in the brain is always preceded by replication in the spleen, lymph nodes and other lymphoreticular tissues (Kimberlin and Walker, 1978). The spleen (especially follicular dendritic cells) (Brown *et al*, 1999; Carp *et al*, 1997; Fraser *et al*, 1996; Lasmézas *et al*, 1996), and lymphoid system including the Peyer's patches in the gut (Maignien *et al*, 1999), and the peripheral nervous system (vegetative and/or sensory) (Groschup *et al*, 1999; McBride and Beekes, 1999) plays a key role in regulating the neuroinvasion of the agent. Genetic asplenia (Dickinson and Fraser, 1972) or splenectomy (Fraser and Dickinson, 1970, 1978) performed before peripheral infection or soon afterwards, lengthens the incubation period of the disease. In contrast, after intracerebral injection of host-adapted agents, splenectomy does not modify the timing of replication of the agent in the brain, and this suggests that this route by-passes the extraneural stage of scrapie pathogenesis (Fraser and Dickinson, 1970). This explains the relatively shorter interval between accidental exposure of the CJD agent and appearance of clinical signs in centrally infected cases of CJD (1-2 years) compared with peripherally infected cases (several years to decades) (Brown, 1988c). After peripheral injection, the scrapie agent is hypothesised to move from the spleen and lymph nodes through retrograde axonal transport in autonomic nerve fibers to the spinal cord or the brain stem, and from here, arrives at the 'clinical target areas' of the brain (Kimberlin and Walker, 1983). When the infectious agent has reached the brain, it replicates at an exponential rate until the appearance of the disease (Kimberlin, 1976; Moreau Dubois *et al*, 1982; Kimberlin and Walker, 1986a; Pocchiari and Masullo, 1988). Each strain of scrapie and CJD produces histological lesions and PrP^{Sc} accumulation in specific brain regions, and this may result, in humans, in the clinical and pathological heterogeneity. In natural infection of scrapie in sheep and goats the spread of the agent follows a pattern similar to that described for the murine model (Hadlow *et al*, 1980, 1982).

IV.4. Conclusions

Important host factors which might influence the risk of developing the disease after exposure to TSE agents include the genetic background of the host, the age, and the route of transmission. In humans, a susceptibility factor is the *PRNP* gene, with the mutations and polymorphism at codon 129 having an important role. It is unknown whether other, especially environmental factors contribute to TSE susceptibility as well. *PRNP* codon 129 may also regulate the age at onset and the duration of disease in familial and sporadic diseases, as well as in kuru. However, the clinical characteristics of human TSEs do not always correlate with the *PRNP* genotype.

V. EFFECT OF ROUTE OF INFECTION ON BSE DOSE: THE RELATIVE EFFICIENCY OF THE ORAL AND THE INTRA-CEREBRAL ROUTES.

There are several possible approaches to the calculation of the relative efficiency of different routes of infection, and hence to calculate an estimate of the effective titre of cattle tissue orally delivered to humans. Risk assessments can be based solely on existing BSE transmission data but since these experiments are incomplete, certain assumptions about titres are made. Alternatively they can encompass information from other TSE transmission experiments.

Data from mouse TSE models show that the intravenous route is approximately 10^1 fold, intra-peritoneal route 10^2 fold, and oral route 10^5 fold less efficient than the i.c. route, at least in some models (Kimberlin and Walker, 1988); i.e. the measured titres are lower by these routes. The relative efficiency of the oral route within species and between species is not known and a trans-species oral route cannot be assumed to be less efficient. Overall the worst case value of 10^8 cow i.c. ID_{50} and of 10^5 fold for the reduction in efficiency of oral BSE transmission to another species are suggested. (See also Kimberlin and Walker, 1978).

Note: To date, the effect of the differences in anatomy and physiology between the digestive tract of ruminants and humans on TSE susceptibility are not known.

V.1. Calculations from the attack rate experiment

Previous opinions provided by the SSC (for example on cross-contamination, September 1998, and on meat-and-bone meal, March 1998) have been based partly on data prepared for BSE risk assessments for or by various UK authorities. These assumed that 1 g of CNS from cattle with BSE at the terminal stage of disease contained approximately 10^1 oral cow ID_{50}/g . The basis for these calculations included the knowledge from the “attack rate experiment” that 1g of brain, and probably less, contained at least 1 cattle oral ID_{50}/g . However an end point was not determined, hence the cattle oral ID_{50}/g is not known, nor is the cattle i.c. titre of the individual or pooled brains used known. It is noted that the pool of brains used in the attack rate study is being titrated i.c. and further dilutions of brain (0.1g, 0.01g and a repeat of 1g) are being used for oral challenges in an extended attack rate study. If we assume that at least some cows in the 0.1g group go down with disease but none in the 0.01g group succumb, an end-point titration will have been achieved. In summary, at present it is not known what the mouse or cattle, i.c. or oral, titres of this inoculum pool are, and

how this relates to the mouse or cattle i.c. titre in other brains from cattle in the epidemic and killed at a similar stage of disease.

Estimated cattle oral dose: 10^1 ID₅₀/g

V.2. Diringer's (1999) calculations

From calculations carried out by Diringer (1999) using the results of published and peer-reviewed experiments, it appears that this value may be 5 times higher (at least 5×10^1 cattle oral ID₅₀/g of cattle CNS tissue). The detail of Diringer's logic approach and corresponding calculations are given in Annex 2. The approach and calculations were discussed in detail and the WG agreed that the results were acceptable.

Estimated cattle oral dose: 5×10^1 ID₅₀/g

V.3. Calculations from BSE transmission experiments to cattle and mice

Information is available from previous titrations of cattle CNS tissue with natural BSE killed well into the clinical phase of the disease: They show that the mouse i.c. titres can vary from 10^3 ID₅₀/g – $10^{5.4}$ ID₅₀/g. The comparative titres in mice and cattle from the same brain source are $10^{3.3}$ ID₅₀/g and $> 10^6$ ID₅₀/g respectively. Titres in different parts of the brain are likely to vary (as they do in natural sheep scrapie (Hadlow, 1980, 1982). There is strongly suggestive evidence of this from PrP^{Sc} distribution studies on different parts of the bovine brain and from titres from whole brain, anterior brain and brainstem used in various other experiments. If the cattle brain pool titre in the attack rate study turns out to be high (e.g. 10^5 mouse i.c. ID₅₀/g), then the SSC assumptions about the low efficiency of oral infection are valid. However, if the titre turns out to be low (e.g. 10^3 mouse i.c. ID₅₀/g), then 1 cow oral ID₅₀ could be contained in 0.001g (i.e. 10^3 cow oral ID₅₀/g) of brainstem from a cow with a titre of 10^5 mouse i.c. ID₅₀/g. Oral transmission to mice was obtained by Barlow and Middleton (1990) and by Middleton and Barlow, (1993) who fed large amounts (1 - 10 g) of brain of unknown titre to mice. Transmission was not obtained using 20mg of BSE brain which had a mouse i.c. titre of $10^{3.4}/20\text{mg}$ i.e. 10^5 mouse i.c. ID₅₀/g, equivalent to 10^8 cow i.c. ID₅₀ (Fraser *et al*, unpublished). It can be calculated that in this experiment 10^6 cow i/c ID₅₀ failed to transmit by the oral route to mice. Oral transmission to sheep has been achieved with 0.5g BSE infected brain of unknown titre. (Foster *et al*, 1993). A worst case could result from exposure to brainstem tissue from a cow with advanced clinical disease in which the titre was variously measured as follows:

- in mice by the i.c. route: 10^5 mouse i.c. ID₅₀/g,
- in cattle by the i.c. route: 10^8 cattle i.c. ID₅₀/g and
- in cattle by the oral route: 10^3 cattle oral ID₅₀/g.

In this example the comparative efficiency of the i.c. to oral route approximates to 10^5 to 1 which is in close agreement with the relative efficiencies of these routes when measured in mice and between cattle and mice (Kimberlin and Walker, 1988, 1989; Kimberlin, 1994).

Schreuder (1998) found ranges from 10^2 to 0.5×10^3 cattle oral ID₅₀/g CNS tissue.

Estimated cattle oral dose: 10^3 ID₅₀/g

V.4. Calculations from all TSE experiments

The above calculations are based primarily on experiments on the transmission of BSE to cattle and mice. However, if the aim of the risk assessment is to estimate the highest possible exposure, data on the transmission of all TSEs are relevant. In this case, an estimate of the highest probable titre in cattle brain is made, based on existing direct measurements in cattle which are incomplete, but also taking into account information from other TSE infectivity measurements. Information on the relative efficiency of other routes to intracerebral injection is also evaluated, again taking into account the limited available data from BSE cattle experiments and the more extensive data from rodent model systems. This approach suggests that the dose to which humans are exposed to per unit mass of tissue may be higher than existing data from BSE transmission experiments suggests.

The most efficient route of TSE infection is by intracerebral injection, when the donor and recipient are from the same species. The available data (see above) suggest that titres in some parts of cattle brains could be as high as 10^8 cow i.c. ID₅₀/g. (The highest recorded TSE titres are 10^{10} hamster i.c.ID₅₀/g for the 263K strain (Kimberlin and Walker, 1977). Data from mouse TSE models show that the intravenous route is approximately 10^1 fold, intra-peritoneal route 10^2 fold, and oral route 10^5 fold less efficient, at least in some models; i.e. the measured titres are lower by these routes. (See for example: Kimberlin *et al*, 1978; Kimberlin and Walker, 1989). The relative efficiency of the oral route within species and between species is not known and a trans-species oral route cannot be assumed to be less efficient. Overall the worst case value of 10^8 cow i.c. ID₅₀ and of 10^5 fold for the reduction in efficiency of oral BSE transmission to another species are suggested.

Estimated cattle oral dose: 10^3 ID₅₀ / g

V.5. Conclusions

Overall these various approaches yield orders of magnitude from 10^1 to 10^3 ID₅₀/g cattle oral doses from a clinically infected brain. The higher value may represent a worst case scenario if the oral route is more efficient than data suggest and that a particularly high titre of infected brain is sampled. Nevertheless the possibility that such a high dose might be encountered cannot be ruled out. The above discussion suggests that the Opinions of the SSC on Cross Contamination and the Safety of MBM may have to be revised on account of the fact that the worst scenario situation is quantitatively worse than that assumed when the Opinions were prepared.

VI. SPECIES BARRIER INCLUDING TRANSGENIC ANIMALS

Transgenic technology has become a central approach to answer questions of the phenomenon of the species barrier. Since *PrP* genes have been found in all mammalian species investigated so far (e.g. Oesch *et al*, 1985; Kretzschmar *et al*, 1986; Goldmann *et al*, 1991) it has become evident that their primary amino acid sequences are highly conserved, but have also variable regions (e.g. the human and the bovine PrP are 93% homologous which vice versa means that around 20 amino acids are different). In the light of the central role of PrP in the disease process, it is obvious to speculate that these differences could potentially account for difficulties in transmission of the TSE

agent between two different species, the species barrier. In transgenic animals this rationale was in the first instance examined by introducing the hamster *PrP* gene into the germline of mice (Scott *et al.*, 1989; Race *et al.*, 1995). These transgenic mice became readily susceptible¹⁹ to the hamster agent and produced the homologous type of agent when infected with material from either hamster or mouse (Prusiner *et al.*, 1990).

The situation was more complicated when the human *PrP* gene (*PRNP*) was introduced into mice. Only after a chimeric gene with murine N and C-termini and the central part of *PRNP* (codons 96 to 167) was expressed in transgenic animals did these mice become susceptible to human prions (Telling *et al.*, 1995). These data are in stark conflict with findings of another group who succeeded in breaking the species barrier by introducing unmodified *PRNP* into mice (Collinge *et al.*, 1995). The latter study also showed that the introduced human transgene construct did not enhance the susceptibility of mice towards BSE comparing the incubation times of such a transmission with a transmission of BSE to normal mice. A problem of this study was the fact that the human transgene carried a valine at the polymorphic codon 129 and bovine PrP has a methionine at this position. At a recent meeting²⁰ the same group reported preliminary results on transgenic mice carrying methionine at codon 129. Again these mice were not more susceptible to BSE than normal mice, a feature that the BSE agent seems to share with the agent causing new variant CJD (vCJD). The similarity of BSE and vCJD was also reported by this group earlier after transmission of both agents to the transgenic line carrying valine at codon 129 (Hill *et al.*, 1997).

In an attempt to produce mice which are more susceptible to the BSE agent, a bovine *PrP* gene was introduced in transgenic mice and these mice were shown to be much more susceptible to the BSE agent than wild-type mice (Scott *et al.*, 1997). Unexpectedly, mice carrying a chimeric mouse/bovine *PrP* gene similar to the chimeric mouse/human mice (Telling *et al.*, 1995), were resistant to the BSE agent (Scott *et al.*, 1997). This has led to the proposal that the crucial epitope in contributing to the species barrier is determined by four amino acids (numbering according to the bovine sequence): No. 195 [valine in cattle, isoleucine in human], 197 [glutamate in cattle, glutamine in human], 214 [isoleucine in both species] and 216 [methionine in cattle and isoleucine in man]. This work has now been repeated (Buschmann, 1999). At the meeting “*Characterization and Diagnosis of Prion Diseases in Animals and Man*” (Tübingen/Germany, 23rd to 25th September 1999), M.Scott and A.Buschmann reported that there seems to be no species barrier in such mice as no shortening in the incubation was observed comparing the first and the second passages. Mike Scott’s experiments revealed furthermore that vCJD behaves exactly like BSE in the

¹⁹ It is important not to confuse “susceptibility” with incubation period. A (transgenic) animal may be more or less susceptible i.e. require more or less inoculum to infect than a control. Independently it may have a shorter or longer incubation period than the control. As far as the Working Group is aware, BSE has not been titrated in any of the transgenic models mentioned. If this is correct, there is no evidence available which demonstrates that any transgenic mice are more susceptible to BSE (i.e. will give higher estimates of BSE titre) than conventional mice. Until such experiments are performed it is not valid to assume that the transgenic mice will be more susceptible.

²⁰ “Characterization and Diagnosis of Prion Diseases in Animals and Man”, Tübingen/Germany, 23rd to 25th September 1999.

transgenic mice carrying a bovine PrP gene adding further evidence to the striking similarity of the BSE and vCJD agents (Scott *et al*, 1999). Since these mice seem to lack a species barrier when challenged with the BSE agent and in addition show a relatively short incubation time, it will be possible in the near future to address a number of important questions such as the titres of infectivity contained in various organs of BSE- infected cattle using a much better system.

However, the conclusion that can be drawn from the Scott *et al* (1999) paper (regarding the species barrier issue) is that no species barrier existed in the primary inoculation, both for “BSE prions” from bovine and from vCJD-patients to *bovine* Tg mice. The paper does not permit to conclude about a species barrier *the other way around*, namely from bovines to humans.

In the transgenic experiments reported by Scott and Buschmann, both vCJD and BSE inocula resulted in similar neuropathology and incubation periods. The fact that no shortening of incubation period occurred between the primary and secondary passage seems indeed highly striking and relevant. However, it does not take into account all other work that has been done with regard to the species barrier, in particular results from the *in vitro* conversion assay. This method has so far provided an opportunity to assess the relative efficiency with which PrP^C of various hosts and various genotypes can be induced by PrP^{Sc} to convert into the pathological form. This method has shown to produce results for the scrapie polymorphisms that follow very closely the real life situation in the field (Bossers *et al*, 1997). Findings with regard to the cattle-human species barrier published by Raymond *et al* in 1997 were also considered. These results indicated an at least 10 fold less efficient conversion of human PrP^C by BSE PrP^{Sc} when compared with the homologous bovine-bovine interaction (3 and 10% for 129-Valine and 129-Methionine, respectively, of the 100% homologous bovine conversion).

These studies on transgenic animals have clearly shown that the species barrier in prion diseases is to a large extent determined by the primary sequence of PrP, while the exact requirements in the PrP sequence for the species barrier are not always fully understood and may vary from one species-to-species transmission to another. However, other factors such as the strain of agent can contribute to the species-barrier effect. With respect to BSE we may now have very good transgenic models which may lack a species barrier, with respect to CJD there are seriously conflicting data in different transgenic systems.

Remark: The Working Group considered the theoretical possibility of a "negative species barrier", but did not find **convincing** evidence to support this concept. **However, recent data from in-vitro conversion experiments do not exclude such a possibility (Bossers *et al*, 2000).**

Conclusions:

The Working Group assumes that by definition, there is a species barrier for animal to human transmission of TSE. The barrier in theory could be high or low. Current evidence suggests that it is unlikely to be absolute but it is not currently possible to quantify it. The barrier appears to be determined by the strain of agent and by the difference between the *PrP* gene sequence of animal donor species and the human recipient. Until the advent of BSE in cattle in 1985/1986 there was no known causal relationship between human TSE of any kind and animal TSE. Thus, a critical factor that has changed this situation is the occurrence of a newly identified TSE agent, the BSE agent.

It is probable that the human *PrP* gene (*PRNP*) is of paramount importance for the determination of the size of this particular species barrier. However, the exact requirements in the *PrP* sequence for the species barrier are not fully understood and may vary from one species-to-species transmission to another. The size of the species barrier for BSE-in-ruminants to BSE-in-humans is not known and may be large (for example a barrier of the order of 1000, as assumed in some risk assessments) or small. **Given the conflicting scientific data and thus the uncertainties about the bovine-to-human species barrier as outlined in this document, the assumption of a worst case scenario considering no (=1) barrier should be included, although available evidence indicates that values greater than 1 are likely to be more realistic.** The Working Group therefore recommends that, until more scientific data are available, for risk assessments of human exposure to potentially BSE contaminated products, a species barrier of about 1 should be considered as a worst case scenario and that, in risk assessments, the range from 10^4 to 10^1 is considered. The latter order of magnitude would imply that the minimal infective dose value(s) considered/accepted to be valid for animals, should also be applied for humans. (See also Section V).

VII. SINGLE DOSE VERSUS REPEATED/CUMULATIVE DOSE:

A key-issue in the exploitation of TSE-related risk assessments is the expected numbers of animals (or humans) that may be exposed to (residual) infectivity in animal material, possibly after processing and/or dilution. Three, simplified, scenarios can be formulated (see also Diringer *et al*, 1998):

1. Although a single dose below the infectious dose will, by definition, not produce infection, repeated exposures to such doses, given at intervals shorter than a certain length, may result in accumulation of infectivity and may eventually possibly achieve an infectious dose;
2. There is a finite probability of infection resulting from a given low dose (below the ID_{50}). However, this probability may be low but could increase in proportion to the number of times the same individual or different individuals are exposed.
3. A single dose is sufficient to cause disease if high enough and that accumulation of infectivity over a period is not a significant factor in achieving a dose that will result in disease.

It is also important to consider the effect of the replication site hypothesis proposed and supported by Dickinson and Outram (1979). If replication sites are already occupied then the consequences of exposure to a different strain of agent may be nil or reduced. This is compatible with “viral interference” and has been reported as strain competition.

Following discussion, the Working Group suggests:

- As it is presently not known if a threshold dose of the TSE agent exists below which there is no risk of establishing an infection and in the absence of knowledge of the shape of the dose-response curve in the low dose range, the Working Group suggests to use the probability scenario in the TSE context. It assumes a linear dose-response curve down to the low dose range. This is a conservative assumption and would mean, as an example, that a product containing an evenly distributed residual

infectivity of 10^{-3} ID₅₀/g and given to each of 1 million individuals, may result in 500 individuals being infected. This number will increase as the number of administrations increases. (This increase, however, will not be linear as individuals who already received a dose, may receive a second, third, fourth, and possibly more doses. The issue in practice would also be difficult to interpret as, where repeated exposures occur, it is most unlikely that all would be of a consistent sub-lethal dose level.)

However, evidence from the BSE epidemic suggests that infectivity in feed is not evenly distributed but occurs in 'packets'. It is therefore important to take into consideration the fact that the TSE agent is very hydrophobic and prone to aggregate. With low doses the repartition of the infectivity may then follow the Poisson distribution.

The dose response relation is not known. Whether the dose/response relationship in the low dose range (for low levels of potential residual infectivity in products after appropriate processing and handling, i.e., after appropriate sourcing, removal of SRMs, processing, avoidance of cross-contamination, etc.) is linear or follows for example a sublinear dose-response relationship or a Poisson distribution, does not immediately affect the outcome of the assessment as such (in terms of absolute numbers of people at risk) but may affect the perception of the risk in management terms. In the first case, a whole (sub-)population is theoretically exposed to a same, but low level of residual infectivity. In the second case, a major part of a population will not be exposed at all to any infectivity, because it is concentrated (aggregated) in a smaller number of consumption units. However, the part of the population that is exposed, will more likely get infected because the infectivity level is higher.

- The accumulative infectivity scenario may also be valid, provided the interval of administration is not too long (probably less than about 2-3 days) and that the repeated doses are sufficiently high so that an infective dose is reached in steady state (the repeated individual doses must be higher than the capacity of an individual to inactivate the infectivity/agent during the interval of administration. From laboratory results (Beringue *et al*, 2000; Diringer *et al*, 1998) it appears that the clearance period is approximately 24-48 hours; beyond that, macrophages are again capable to take up their clearance function. Further research in this area is urgently needed.

Remark: The Working Group is aware of the fact that, according to certain scientists, the scenario of infectivity being accumulated (the cumulative dose effect) has not been proved. In that (third) scenario (no cumulative dose), significant numbers of additional cases would not result.

Conclusions:

In practice, in terms of risk assessment, the risks resulting from (low) residual infectivity should, at present - until further evidence is available - be calculated as fractions of a population that would be exposed and *possibly* get infected. In addition,

repeated exposure would possibly increase the risk both in absolute number of cases and the likelihood that exposure would result in *effective* infection.

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IX. LITERATURE CONSULTED

- AGRIMI U., RU G., CARDONE, F., POCCHIARI, M, CARAMELLI, M., 1999.** Epidemic of transmissible spongiform encephalopathy in sheep and goats in Italy. *The Lancet* **353**, 560-561
- 1.1.1.* **AGUZZI, A., 1998.** Protein conformation dictates prion strain. *Nature Medicine*, **4**, 1125-1126.
- AGUZZI, A., COLLINGE, J., 1997.** Post-exposure prophylaxis after accidental prion inoculation. *Lancet* **350**, 1519-1520.
- ALMER, G., HAINFELLNER, J. A., BRÜCKE, T., JELLINGER, K., KLEINERT, R., BAYER, G., WINDL, O., KRETZSCHMAR, H. A., HILL, A., SIDLE, K., COLLINGE, J., BUDKA, H., 1999.** Fatal familial insomnia: a new Austrian family. *Brain* **122**, 5-16.
- ALPEROVITCH, A., ZERR, I., POCCHIARI, M., MITROVA, E., CUESTA, J. D. P., HEGYI, I., COLLINS, S., KRETZSCHMAR, H., DUIJN, C. V., WILL, R.G., 1999.** Codon 129 prion protein genotype and sporadic Creutzfeldt-Jakob disease. *Lancet* **353**, 1673-1674.
- APC EUROPE, 2000.** Risk assessment: Spray-dried plasma for animal consumption - BSE. Draft Biosafety Manual. Barcelona, 45 pp.
- BARLOW, R.M., MIDDLETON, D. J., 1990.** Dietary transmission of bovine spongiform encephalopathy to mice. *Vet Rec* **126**, 111-112.
- BERINGUE V, DEMOY M, LASMEZAS CI, GOURITIN B, WEINGARTEN C, DESLYS JP, ANDREUX JP, COUVREUR P, DORMONT D, 2000.** Role of spleen macrophages in the clearance of scrapie agent at early stages of infection. *Journal of Pathology*, **190**, 495-502.
- BESSEN, R.A., MARSH, R.F. 1994.** Distinct PrP properties suggest the molecular basis of strain variation in TME. *J Virol*. **68**, 7859-7868.
- BILLETTE DE VILLEMEUR, T., GOURMELEN, M., BEAUVAIS, P., RODRIGUEZ, D., VAUDOUR, G., DESLYS, J.P., DORMONT, D., RICHARD, P., RICHARDET, J.M., 1992.** Creutzfeldt-Jakob disease in 4 children treated with growth hormone. *Rev Neurol* **148**, 328-334.
- BOSSERS A. ET AL, 1997.** Scrapie susceptibility-linked polymorphisms modulate the in vitro conversion of sheep prion protein to protease-resistant forms. *Proc Natl Acad Sci USA* **94**, 4931-4936
- BOSSERS, A., DE VRIES, R., SMITS, M.A., 2000:** Susceptibility of sheep for scrapie as assessed by in vitro conversion of nine naturally occurring variants of PrP. *J Virol* **74**: 1407-1414.
- BRADLEY R., WILESMITH J.W., 1993.** Epidemiology and control of bovine spongiform encephalopathy (BSE). *British Medical Bulletin*, **49**, 932-959.
- BRADLEY, R., 1996.** Experimental transmission of bovine spongiform encephalopathy. In: transmissible subacute spongiform encephalopathies: prion diseases. L Court,B, Dodet eds. Elsevier, Paris. 51-56.
- BROWN, K. L., STEWART, K., RITCHIE, D. L., MABBOTT, N. A., WILLIAMS, A., FRASER, H., MORRISON, W. I., BRUCE, M.E., 1999.** Scrapie replication in lymphoid tissues depends on prion protein-expressing follicular dendritic cells. *Nature Med* **5**, 1308-1312.
- BROWN, P., GIBBS, C.J., JR., RODGERS JOHNSON, P., ASHER, D.M., SULIMA, M. P., BACOTE, A., GOLDFARB, L. G., GAJDUSEK, D.C., 1994.** Human spongiform encephalopathy: the National Institutes of Health series of 300 cases of experimentally transmitted disease. *Ann Neurol* **35**, 513-529.
- BROWN, P., GOLDFARB, L.G., KOVANEN, J., HALTIA, M., CATHALA, F., SULIMA, M., GIBBS, C.J., JR., GAJDUSEK, D.C., 1992.** Phenotypic characteristics of familial

- Creutzfeldt-Jakob disease associated with the codon 178Asn PRNP mutation. *Ann Neurol* **31**, 282-285.
- BRUCE ME., WILL RG., IRONSIDE JW., McCONNELL I., DRUMMOND D., SUTTLE A., et al., 1997.** Transmissions to mice indicate that 'new variant' CJD is caused by the CJD agent. *Nature*, 389, 489-501.
- BRUCE, M. E., 1985.** Agent replication dynamics in a long incubation period model of mouse scrapie. *J. Gen. Virol.* **66**: 2517-2522.
- BRUCE, M.E., 1994.** Bovine spongiform encephalopathy: experimental studies. In OIE/WHO Consultation on BSE. Paris, OIE, 1994.
- BRUCE, M.E., 1996.** Strain typing studies of scrapie and BSE. In: *Methods in Molecular Medicine: Prion Diseases*, H Baker and R M Ridley Eds. Humana Press Inc. Totowa NJ. Pp 223-236.
- BRUCE, M.E., DICKINSON, A.G., 1987.** Biological evidence that scrapie agent has an independent genome. *J. Gen. Virol.* **68**: 79-89.
- BUDKA, H., 1998.** Iatrogene Creutzfeldt-Jakob-Krankheit. *Wien Klin Wochenschr* **110**, 451-454.
- BUSCHMANN, A., 1999.** Detection of cattle-derived BSE prions using transgenic mice overexpressing PrP^C. In: *Proceedings of the Conference "Characterization and Diagnosis of Prion Diseases in Animals and Man"*. Tübingen/Germany, 23-25 September 1999.
- BUTLER, D.A, SCOTT, M.R.D., BOCKMAN, J.M., BORCHELT, D.R., TARABOULOS, A., HSIAO, K.K., KINGSBURY, D.T., PRUSINER, S.B., 1988.** Scrapie-infected murine neuroblastoma cells produce protease-resistant prion protein. *Journal of Virology*, 62(5), 1558-1564.
- CAPUCCHIO MT, GUARDA F, ISAIA MC, CARACAPPA S, DI MARCO, V., 1998.** Natural occurrence of scrapie in goats in Italy. *The Veterinary Record*, **143**, 452-453
- CARP, R. I., MEEKER, H., SERSEN, E., 1997.** Scrapie strains retain their distinctive characteristics following passages of homogenates from different brain regions and spleen. *J Gen Virol* **78**, 283-290.
- CARP, R.I., KIMBERLIN, R.H., 1991.** Scrapie-infected spleens: analysis of infectivity, scrapie-associated fibrils, and protease-resistant proteins." *Journal of Infectious Diseases* **164**(1): 29-35.
- CERVENAKOVA, L., BUETEFISCH, C., LEE, H. S., TALLER, I., STONE, G., GIBBS, C.J.J., BROWN, P., HALLETT, M., GOLDFARB, L.G., 1999.** Novel PRNP sequence variant associated with familial encephalopathy. *Am J Med Genet* **88**, 653-656.
- CERVENAKOVA, L., GOLDFARB, L. G., GARRUTO, R., LEE, H. S., GAJDUSEK, D. C., BROWN, P., 1998.** Phenotype-genotype studies in kuru: Implications for new variant Creutzfeldt-Jakob disease. *Proc Natl Acad Sci USA* **95**, 13239-13241.
- CHAPMAN, J., BEN ISRAEL, J., GOLDHAMMER, Y., KORCZYN, A. D. (1994):** The risk of developing Creutzfeldt-Jakob disease in subjects with the PRNP gene codon 200 point mutation. *Neurology* **44**, 1683-1686.
- CHESEBRO, B. (1999):** Minireview: Prion protein and the transmissible spongiform encephalopathy diseases. *Neuron* **24**, 503-506.
- COCHRAN, E. J., BENNETT, D. A., CERVENAKOVA, L., KENNEY, K., BERNARD, B., FOSTER, N. L., BENSON, D. F., GOLDFARB, L. G., BROWN, P., 1996.** Familial Creutzfeldt-Jakob-disease with a 5-repeat octapeptide insert mutation. *Neurology* **47**, 727-733.
- COLLINGE J., SIDLE, K.C.L., MEADS, J., IRONSIDE, J., HILL, A.F. 1996.** Molecular analysis of prion strain variation and the aetiology of new variant CJD. *Nature*, **383**, 685-690.
- COLLINGE, J., 1999.** Variant Creutzfeldt-Jakob disease. *Lancet* **354**, 317-323.
- COLLINGE, J., PALMER, M. S., DRYDEN, A.J., 1991.** Genetic predisposition to iatrogenic Creutzfeldt-Jakob disease. *Lancet* **337**, 1441-1442.
- COLLINGE, J., PALMER, M.S., CAMPBELL, T., SIDLE, K. C., CARROLL, D., HARDING, A., 1993.** Inherited prion disease (PrP lysine 200) in Britain: two case reports. *Brit Med J* **306**, 301-302.

- COLLINGE, J., PALMER, M.S., SIDLE, K.C.L., HILL, A.F., GOWLAND, I., MEADS, J., ASANTE, E., BRADLEY, R., DOEY, L.J., LANTOS, P.L., 1995.** Unaltered susceptibility to BSE in transgenic mice expressing human prion protein. *Nature* **378**, 779-783.
- COLLIS, C., KIMBERLIN, R.H., 1985.** Long term persistence of scrapie infection in mouse spleens in the absence of clinical disease. *FEMS Microbiology Letters*, **29**, 111-114.
- COUSENS, S. N., ZEIDLER, M., ESMONDE, T. F., DE SILVA, R., WILESMITH, J. W., SMITH, P. G., WILL, R.G., 1997.** Sporadic Creutzfeldt-Jakob disease in the United Kingdom: analysis of epidemiological surveillance data for 1970-96. *Brit Med J* **315**, 389-395.
- DESLYS, J. P., MARCÉ, D., AND DORMONT, D., 1994.** Similar genetic susceptibility in iatrogenic and sporadic Creutzfeldt-Jakob disease. *J Gen Virol* **75**, 23-27.
- DESLYS, J.-P., JAEGLY, A., D'AIGNAUX, J. H., MOUTHON, F., BILLETTE-DEVILLEMEUR, T., DORMONT, D., 1998.** Genotype at codon 129 and susceptibility to Creutzfeldt-Jakob disease. *Lancet* **351**, 1251.
- DICKINSON, A. G., FRASER, H., OUTRAM, G. W., 1975.** Scrapie incubation time can exceed natural lifespan. *Nature* **256**, 732-733.
- DICKINSON, A.G, TAYLOR, D.M., 1988.** Options for the control of scrapie in sheep and its counterpart in cattle. *Proc 3rd Int Congress on sheep and cattle breeding.* 16-19 June 1988, Paris.
- DIRINGER, H., 1999.** Bovine spongiform encephalopathy (BSE) and public health. In: Aggett, P.J., Kuiper, H.A. (Eds), 1999. Risk assessment in the food chain of children. Nestlé Nutrition Workshop Series, **44**, 225-233. Nestlé Ltd., Vevey/Lippincott Williams & Wilkins Publishers, Philadelphia.
- DIRINGER, H., ROEHMEL, J., BEEKES, M., 1998.** Effect of repeated oral infection of hamsters with scrapie. *J. gen. Virol.*; **79**: 609-612.
- FOSTER JD., BRUCE M., McCONNELL I., CHREE A., FRASER H., 1996.** Detection of BSE infectivity in brain and spleen of experimentally infected sheep. *Veterinary Record*, **138**, 546-548.
- FOSTER, J. D., HOPE, J., FRASER, H., 1993.** Transmission of bovine spongiform encephalopathy to sheep and goats. *Vet Rec* **133**, 339-341.
- FRASER, H., BROWN, K. L., STEWART, K., MCCONNELL, I., MCBRIDE, P., WILLIAMS, A., 1996.** Replication of scrapie in spleens of SCID mice follows reconstitution with wild-type mouse bone-marrow. *J Gen Virol* **77**, 1935-1940.
- FRASER, H., BRUCE, M. E., CHREE, A., MCCONNELL, I., WELLS, G.A., 1992.** Transmission of bovine spongiform encephalopathy and scrapie to mice. *J Gen Virol* **73**, 1891-7.
- GAMBETTI, P., PARCHI, P., PETERSEN, R. B., CHEN, S. G., LUGARESI, E., 1995.** Fatal familial insomnia and familial Creutzfeldt-Jakob disease: clinical, pathological and molecular features. *Brain Pathol* **5**, 43-51.
- GHETTI, B., DLOUHY, S. R., GIACCONE, G., BUGIANI, O., FRANGIONE, B., FARLOW, M. R., TAGLIAVINI, F., 1995.** Gerstmann-Straussler-Scheinker disease and the Indiana kindred. *Brain Pathol* **5**, 61-75.
- GOLDMANN, W., CHONG, A., FOSTER, J., HOPE, J., HUNTER, N., 1998.** The shortest known prion protein gene allele occurs in goats has only three octapeptide repeats and is non-pathogenic. *Journal of General Virology*, **79**, 3173-3176.
- GOLDMANN, W., HUNTER, N., MARTIN, T., DAWSON, M., HOPE, J., 1991.** Different forms of the bovine PrP gene have five or six copies of a short, G-C-rich element within the protein-coding exon. *J.Gen.Virol.* **72**, 201-204.
- GOLDMANN, W., HUNTER, N., SOMERVILLE, R., HOPE, J. 1996.** Prion phylogeny revisited. *Nature*, **382**, 32-33.

- GOLDMANN, W., MARTIN, T., FOSTER, J., HUGHES, S., SMITH, G., HUGHES, K., DAWSON, M., HUNTER, N., 1996.** Novel polymorphisms in the caprine PrP gene: a codon 142 mutation associated with scrapie incubation period. *Journal of General Virology*, 77, 2885-2891.
- GROBET, L., VANDEVENNE, S., CHARLIER, C., PASTORET, P.P., HANSET, R., 1994.** Polymorphisme du gène de la protéine prion chez des bovins belges. *Annales Médecine Vétérinaire*, 138, 581-586.
- GROSCHUP, M. H., BEEKES, M., MCBRIDE, P. A., HARDT, M., HAINFELLNER, J. A., BUDKA, H., 1999.** Deposition of disease-associated prion protein involves the peripheral nervous system in experimental scrapie. *Acta neuropathol* 98, 453-457.
- HADLOW W.J., KENNEDY R.C., RACE R.E., 1982.** Natural infection of Suffolk sheep with scrapie virus. *Journal of Infectious Diseases*, 146, 657-664.
- HADLOW W.J., KENNEDY R.C., RACE R.E., EKLUND C.M., 1980.** Virological and neurohistological findings in dairy goats affected with natural scrapie. *VeterinaryPathology*, 17, 187-199.
- HADLOW W.J., RACE R.E., KENNEDY R.C., EKLUND C.M., 1979.** Natural infection of the sheep with scrapie virus. In: PRUSINER, S.B & HADLOW W.J., (eds)., *Slow transmissible diseases of the nervous system*. Vol 2. Academic Press, New York. Pp. 3-12.
- HAINFELLNER, J. A., BRANTNER-INTHALER, S., CERVENAKOVA, L., BROWN, P., KITAMOTO, T., TATEISHI, J., DIRINGER, H., LIBERSKI, P. P., REGELE, H., FEUCHT, M., MAYR, N., WESSELY, P., SUMMER, K., SEITELBERGER, F., BUDKA, H., 1995.** The original Gerstmann-Sträussler-Scheinker family of Austria: divergent clinicopathological phenotypes but constant PrP genotype. *Brain Pathol* 5, 201-211.
- HAINFELLNER, J. A., BUDKA, H., 1999.** Disease associated prion protein may deposit in the peripheral nervous system in human transmissible spongiform encephalopathies. *Acta Neuropathol* 98, 458-460.
- HAINFELLNER, J. A., JELLINGER, K., DIRINGER, H., GUENTCHEV, M., KLEINERT, R., PILZ, P., MAIER, H., BUDKA, H., 1996.** Creutzfeldt-Jakob disease in Austria. *J Neurol Neurosurg Psychiat* 61, 139-142.
- HALTIA, M., KOVANEN, J., GOLDFARB, L. G., BROWN, P., GAJDUSEK, D.C., 1991.** Familial Creutzfeldt-Jakob disease in Finland: epidemiological, clinical, pathological and molecular genetic studies. *Eur-J-Epidemiol* 7, 494-500.
- HAU C.M., CURNOW R.N., 1996.** Separating the environmental and genetic factors that may be causes of bovine spongiform encephalopathy. *Philosophical Transactions of the Royal Society London B*. 351, 913-920.
- HILL AF., SIDLE KCL., JOINER S., KEYES P., MARTIN TC., DAWSON M., COLLINGE J. 1998.** Molecular screening of sheep for bovine spongiform encephalopathy. *Neuroscience Letters*, 255, 159-162.
- HILL, A. F., BUTTERWORTH, R. J., JOINER, S., JACKSON, G., ROSSOR, M. N., THOMAS, D. J., FROSH, A., TOLLEY, N., J E BELL, SPENCER, M., KING, A., AL-SARRAJ, S., IRONSIDE, J. W., LANTOS, P. L., COLLINGE, J., 1999.** Investigation of variant Creutzfeldt-Jakob disease and other human prion diseases with tonsil biopsy samples. *Lancet* 353, 183-189.
- HILL, A. F., ZEIDLER, M., IRONSIDE, J., COLLINGE, J., 1997.** Diagnosis of new variant Creutzfeldt-Jakob disease by tonsil biopsy. *Lancet* 349, 99-100.
- HILL, A.F., ANTONIOU, M., COLLINGE, J., 1999.** Protease-resistant prion protein produced in vitro lacks detectable infectivity. *J. Gen.Virol.*, 80, 11-14.
- HILL, A.F., DESBRUSLAIS, S.J., JOINER, S., SIDLE, K.C.L., GOWLAND, I., COLLINGE, J., DOEY, L.J., LANTOS, P., 1997.** The same prion strain causes vCJD and BSE. *Nature*, 389, 448-450.

- HILTON, D. A., FATHERS, E., EDWARDS, P., IRONSIDE, J. W., ZAJICEK, J., 1998.** Prion immunoreactivity in appendix before clinical onset of variant Creutzfeldt-Jakob disease (letter). *Lancet* **352**, 703-704.
- HOLMAN, R. C., KHAN, A. S., BELAY, E. D., SCHONBERGER, L. B., 1996.** Creutzfeldt-Jakob disease in the United States, 1979-1994: using national mortality data to assess the possible occurrence of variant cases. *Emerg Infect Dis* **2**, 333-337.
- HOPE J., REEKIE L.J.D., HUNTER N., MULTHAUP G., BEYREUTHER K et al, 1988.** Fibrils from brains of cows with new cattle disease contain scrapie-associated protein. *Nature*, 336, 390-392.
- HOPE, J., WOOD, S.C.E.R., BIRKETT, C.R., CHONG, A., BRUCE, M.E., CAIRNS, D., GOLDMANN, W., HUNTER, N., BOSTOCK, C.J., 1999.** Molecular analysis of ovine prion protein identifies similarities between BSE and an experimental isolate of natural scrapie CH1641. *J Gen Virol*, **80**, 1-4.
- HUNTER, N., GOLDMANN, W., SMITH, G., HOPE, J., 1994.** Frequencies of PrP gene variants in healthy cattle and cattle with BSE in Scotland. *Veterinary Record*, **135**, 400-403.
- KAWASHIMA, T., FURUKAWA, H., DOH-URA, K., IWAKI, T., 1997.** Diagnosis of new variant Creutzfeldt-Jakob disease by tonsil biopsy. *Lancet* **350**, 68-69.
- KIMBERLIN, R. H., H. J. FIELD, ET AL, 1983.** Pathogenesis of mouse scrapie: evidence for spread of infection from central to peripheral nervous system. *J. Gen. Virol.* **64 Pt 3**: 713-716.
- KIMBERLIN, R. H., WALKER, C. A., 1978.** Pathogenesis of mouse scrapie: effect of route of inoculation on infectivity titres and dose-response curves. *Journal of Comparative Pathology.* **88**, 39-47.
- KIMBERLIN, R.H., 1994.** A Scientific Evaluation of Research into Bovine Spongiform Encephalopathy (BSE); in Bradley R, Marchant B: Transmissible spongiform encephalopathies. Proceedings of a consultation with the Scientific Veterinary Committee of the CEC 14-15 September 1993. VI/4131/94-EN Brussels EC 1994; pp 455-477.
- KIMBERLIN, R.H., COLE, S., WALKER, C.A., 1987.** Temporary and permanent modifications to a single strain of mouse scrapie on transmission to rats and hamsters. *J gen Virol*, **68**, 1875-1881.
- KIMBERLIN, R.H., WALKER, C.A., 1988.** Pathogenesis of experimental scrapie; in Bock G, Marsh J (eds): Novel infectious agents and the central nervous system. Ciba Foundation Symposium; Wiley, Chichester, **135**, 37-62.
- KIMBERLIN, R.H., WALKER, C.A., 1989.** Pathogenesis of scrapie in mice after intragastric infection. *Vir Res* **12**, 213-220.
- KIMBERLIN, R.H., WALKER, C.A., ET AL, 1989.** The genomic identity of different strains of mouse scrapie is expressed in hamsters and preserved on reisolation in mice. *Journal of General Virology* **70**, 2017-2025.
- KIMBERLIN, R.H., WALKER, C.A., 1977.** Characteristics of a short incubation model of scrapie in the golden hamster. *J. Gen. Virol.* **34(2)**: 295-304.
- KIRKWOOD, J.K., CUNNINGHAM, A.A., 1994.** Epidemiological observations on spongiform encephalopathies in captive wild animals in the British Isles. *Vet Rec.* **135**, 296-303.
- KLEIN, M. A., FRIGG, R., FLECHSIG, E., RAEBER, A. J., KALINKE, U., BLUETHMANN, H., BOOTZ, F., SUTER, M., ZINKERNAGEL, R. M., AGUZZI, A., 1997.** A crucial role for B cells in neuroinvasive scrapie. *Nature* **390**, 687-690.
- KLEIN, M. A., FRIGG, R., RAEBER, A. J., FLECHSIG, E., HEGYI, I., ZINKERNAGEL, R. M., WEISSMANN, C., AGUZZI, A., 1998.** PrP expression in B lymphocytes is not required for prion neuroinvasion. *Nat Med* **4**, 1429-1433.
- KOCISKO, D.A., COME, J.H., PRIOLA, S.A., CHESEBRO, B., RAYMOND, G.J., LASBURG, P.T., CAUGHEY, B., 1999.** Cell-free formation of protease-resistant prion protein. *Nature*, **370**, 471, 474.

- KRETZSCHMAR, H.A., STOWRING, L.E., WESTAWAY, D., STUBBLEBINE, W.H., PRUSINER, S.B., DEARMOND, S.J., 1986.** Molecular cloning of a human prion protein cDNA. *DNA* **5**, 315-324.
- KURODA, Y., GIBBS, C. J. J., AMYX, H. L., 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.
- LAPLANCHE, J.L., DELASNERIE LAUPRETRE, N., BRANDEL, J.P., CHATELAIN, J., BEAUDRY, P., ALPEROVITCH, A., LAUNAY, J.M., 1994.** Molecular genetics of prion diseases in France. French Research Group on Epidemiology of Human Spongiform Encephalopathies. *Neurology* **44**, 2347-51.
- LAPLANCHE, J.-L., HACHIMI, K. H. E., DURIEUX, I., FONCIN, J.-F., AND DESTÉE, A., 1999.** Prominent psychiatric features and early onset in an inherited prion disease with a new insertional mutation in the prion protein gene. *Brain* **122**, 2375-2386.
- LASMÉZAS, C. I., CESBRON, J. Y., DESLYS, J. P., DEMAIMAY, R., ADJOU, K. T., RIOUX, R., LEMAIRE, C., LOCHT, C., DORMONT, D., 1996.** Immune system-dependent and -independent replication of the scrapie agent. *J Virol* **70**, 1292-1295.
- LASMEZAS, C. I., DESLYS, J., ROBAIN, O., JAEGLY, A., BERINGUE, V., PEYRIN, J., FOURNIER, J., HAUW, J., ROSSIER, J., DORMONT, D., 1997.** Transmission of the BSE agent to mice in the absence of detectable abnormal prion protein. *Science* **275**, 402-405.
- LASMEZAS, C.I., DESLYS, J.P., DEMAIMAY, R., ADJOU, K.T., HAUW, J.J., DORMONT, D., 1996.** Scrapie-specific and common pathogenic events in murine models of scrapie and bovine spongiform encephalopathy. *Journal for General Virology* **77**, 1601-1609.
- MAIGNIEN, T., LASMÉZAS, C. I., BERINGUE, V., DORMONT, D., DESLYS, J.-P., 1999.** Pathogenesis of the oral route of infection of mice with scrapie and bovine spongiform encephalopathy agents. *J Gen Virol* **80**, 3035-3042.
- MAINGIEN, T., LASMÉZAS C.I., BERINGUE, V., DORMONT, D., DESLYS, J-P., 1999.** Pathogenesis of the oral route of infection of mice with scrapie and bovine spongiform encephalopathy agents. *Journal of general Virology*, **80**, 3035-3042.
- MANSON, J.C., JAMIESON, E., BAYBUTT, H., TUZI, N.L., BARRON, R., MCCONNELL, I., SOMERVILLE, R., IRONSIDE, J., WILL, R., MAN-SUN SY, MELTON, D.W., HOPE, J., BOSTOCK, C., 1999.** A single amino acid alteration (101L) introduced into murine PrP dramatically alters incubation time of transmissible spongiform encephalopathy. *The EMBO Journal*, **18**, 6855-6864,
- MASULLO, C., POCCHIARI, M., NERI, G., CASACCIA, P., IAVARONE, A., LADOGANA, A., MACCHI, G., 1988.** A retrospective study of Creutzfeldt-Jakob disease in Italy (1972-1986). *Eur J Epidemiol* **4**, 482-487.
- MCBRIDE, P.A., AND BEEKES, M. 1999.** Pathological PrP is abundant in sympathetic and sensory ganglia of hamsters fed with scrapie. *Neurosci Lett* **265**, 135-138.
- McKENZIE, D.I., COWAN, C.M., MARSH, R.F., AIKEN J.M., 1992.** PrP gene variability in the US cattle population. *Animal Biotechnology*, **3**, 309-315.
- MIDDLETON, D.J., BARLOW, R. M., 1993.** Failure to transmit bovine spongiform encephalopathy to mice by feeding them with extraneural tissues of affected cattle. *Vet. Rec.* **132**, 545-547.
- OESCH, B., WESTAWAY, D., WÄLCHLI, M., MCKINLEY, M.P., KENT, S.B.H., AEBERSOLD, R., BARRY, R.A., TEMPST, P., TEPLow, D.B., HOOD, L.E., PRUSINER, S.B., WEISSMANN, C., 1985.** A cellular gene encodes scrapie PrP 27-30 protein. *Cell* **40**, 735-746.
- OUTRAM, G. W., DICKINSON, A. G., FRASER, H., 1973.** Developmental maturation of susceptibility to scrapie in mice. *Nature* **241**, 536-537.
- PALMER, M. S., DRYDEN, A. J., HUGHES, J. T., COLLINGE, J. (1991):** Homozygous prion protein genotype predisposes to sporadic Creutzfeldt-Jakob disease. *Nature* **352**, 340-342.

- PALS, P., VAN-EVERBROECK, B., SCIOT, R., GODFRAIND, C., ROBBERECHT, W., DOM, R., CLATERRE, MARTIN, J. J., CRAS, P., 1999.** A retrospective study of Creutzfeldt-Jakob disease in Belgium. *Eur J Epidemiol* **15**, 517-519.
- PARCHI, P., GIESE, A., CAPELLARI, S., BROWN, P., SCHULZ-SCHAEFFER, W., WINDL, O., ZERR, I., BUDKA, H., KOPP, N., PICCARDO, P., POSER, S., ROJANI, A., STREICHEMBERGER, N., JULIEN, J., VITAL, C., GHETTI, B., GAMBETTI, P., KRETZSCHMAR, H., 1999.** Classification of sporadic Creutzfeldt-Jakob disease based on molecular and phenotypic analysis of 300 subjects. *Ann Neurol* **46**, 224-233.
- POIDINGER M., KIRKWOOD J., ALMOND J.W., 1993.** Sequence analysis of the prion protein from two species of antelope susceptible to TSE. *Archives of Virology*, **131**, 193-199.
- POULTER, M., BAKER, H. F., FRITH, C. D., LEACH, M., LOFTHOUSE, R., RIDLEY, R. M., SHAH, T., OWEN, F., COLLINGE, J., BROWN, J., ET AL., 1992.** Inherited prion disease with 144 base pair gene insertion. 1. Genealogical and molecular studies. *Brain* **115**, 675-685.
- PRIOLA, S.A., 1999.** Prion protein and species barriers in the TSE. *Biomed & Pharmacother* **53**, 27-33.
- PRUSINER, S.B., 1982.** Novel infectious proteins cause scrapie. *Science* **216**, 136-144.
- PRUSINER, S.B., SCOTT, M., FOSTER, D., PAN, K.M., GROTH, D., MIRENDA, C., TORCHIA, M., YANG, S.-L., SERBAN, D., CARLSON, G.A., HOPPE, P.C., WESTAWAY, D., DEARMOND, S.J., 1990.** Transgenic studies implicate interactions between homologous PrP isoforms in scrapie prion replication. *Cell* **63**, 673-686.
- RACE RE, FADNESS LH AND CHESEBRO B, 1987.** Characterization of scrapie infection in mouse neuroblastoma cells. *Journal of General Virology*, **68**, 1391-1399.
- RACE, R.E., PRIOLA, S.A., BESSEN, R.A., ERNST, D., DOCKTER, J., RALL, G.F., MUCKE, L., CHESEBRO, B., OLDSTONE, M.B.A., 1995.** Neuron-specific expression of a hamster prion protein minigene in transgenic mice induces susceptibility to hamster scrapie agent. *Neuron* **15**, 1183-1191.
- RAEBER, A. J., KLEIN, M. A., FRIGG, R., FLECHSIG, E., AGUZZI, A., AND WEISSMANN, C., 1999.** PrP-dependent association of prions with splenic but not circulating lymphocytes of scrapie-infected mice. *EMBO J* **18**, 2702-2706.
- RAYMOND G.J. ET AL, 1997.** Molecular assessment of the potential transmissibilities of BSE and scrapie to humans. *Nature* **388**, 285-288.
- RAYMOND, G. J., HOPE, J., KOCISKO, D.A., PRIOLA, S.A., RAYMOND, L.D., BOSSERS, A., IRONSIDE, J., WILL, R.G., CHEN, S.G., PETERSEN, R.B., GAMBETTI, P., RUBENSTEIN, R., SMITS, M. A., LANSBURY, P.T.J., CAUGHEY, B., 1997.** Molecular assessment of the potential transmissibilities of BSE and scrapie in humans. *Nature*, **388**, 285-288.
- ROBINSON M.M., CHEEVERS W.P., BURGER D., GORHAM J., 1990.** Organ –specific modification of the dose response relationship of scrapie infectivity. *Journal of Infectious Diseases*, **161**, 783-786.
- RUBENSTEIN, R., MERZ, P.A., KASCSAK, R.J., SCALICI, C.L., PAPINI, M.C. CARP, R.I., KIMBERLIN R.H., 1991.** Scrapie-infected spleens: analysis of infectivity, scrapie associated fibrils and protease resistant protein. *J. Inf Dis.*, **164**, 29-35.
- SAFAR J., 1998.** Evidence that eight prion strains possess PrP^{Sc} molecules with different conformations. *Nature Medicine*, **4**, 1157-1165.
- SAFAR, J., WILLE, H., ITRI, V., GROTH, D., SERBAN, H., TORCHIA, M., COHEN, F. E., PRUSINER, S.B., 1998.** Eight prion strains have PrP(Sc) molecules with different conformations. *Nature Medicine* **4**, 1157-65.
- SALVATORE, M., GENUARDI, M., PETRAROLI, R., MASULLO, C., D'ALESSANDRO, M., POCCHIARI, M., 1994.** Polymorphisms of the prion protein gene in Italian patients with Creutzfeldt-Jakob disease. *Hum Genet* **94**, 375-379.

- SCHREUDER B.E.C., 1994.** Agent strain hypotheses. *Livestock Production Science*, 38,23-33.
- SCHREUDER, B.E.C., GEERTSMA, R.E., VANKEULEN, L.J.M., VANASTEN, J.A.A.M., ENTHOVEN, P., OBERTHUR, R. C., DE KOEIJER, A.A., OSTERHAUS, A.D. M. E., 1998.** Studies on the efficacy of hyperbaric rendering procedures in inactivating bovine spongiform encephalopathy (BSE) and scrapie agents. *Vet Rec* **142**, 474-480.
- SCHREUDER, B.E.C., 1998.** Epidemiological aspects of BSE and scrapie including a risk assessment study. PhD Thesis, Utrecht, ISBN. 90-393-1636-8, page 210
- SCOTT, M., FOSTER, D., MIRENDA, C., SERBAN, D., COUFAL, F., WÄLCHLI, M., TORCHIA, M., GROTH, D., CARLSON, G., DEARMOND, S.J., WESTAWAY, D., PRUSINER, S.B., 1989.** Transgenic mice expressing hamster prion protein produce species-specific scrapie infectivity and amyloid plaques. *Cell* **59**, 847-857.
- SCOTT, M.R., SAFAR, J., TELLING, G., NGUYEN, O., GROTH, D., TORCHIA, M., KOEHLER, R., TREMBLAY, P., WALTHER, D., COHEN, F.E., DEARMOND, S.J., PRUSINER, S.B., 1997.** Identification of a prion protein epitope modulating transmission of bovine spongiform encephalopathy prions to transgenic mice. *Proc Natl Acad Sci USA*, **94**, 14279-14284.
- SCOTT, M.R., WILL, R., IRONSIDE, J., NGUYEN, H-O.B., TREMBLAY, P., DeARMOND, S.J., PRUSINER, S.B., 1999.** Compelling transgenetic evidence for transmission of bovine spongiform encephalopathy prions to humans. *Proc Natl Acad Sci USA*, **96** (26): 15137-15142.
- SEAC 1994.** Transmissible spongiform encephalopathies. A summary of present knowledge and research. HMSO, London p 18.
- SPUDICH, S., MASTRIANNI, J. A., WRENSCH, M., GABIZON, R., MEINER, Z., KAHANA, I., ROSENMANN, H., KAHANA, E., PRUSINER, S.B., 1995.** Complete penetrance of Creutzfeldt-Jakob disease in Libyan Jews carrying the E200K mutation in the prion protein gene. *Mol Med* **1**, 607-613.
- TAYLOR, D. M., S. L. WOODGATE, ET AL, 1995.** Inactivation Of the Bovine Spongiform Encephalopathy Agent By Rendering Procedures." *Veterinary Record* **137**(24): 605-610.
- TAYLOR, D.M., FRASER, H., MCCONNELL, I., BROWN, D. A., BROWN, K. L., LAMZA, K.A., SMITH, G. R. A., 1994.** Decontamination studies with the agents of bovine spongiform encephalopathy and scrapie. *Archives of Virology* **139**, 313-326.
- TAYLOR, D.M., WOODGATE, S.L., ATKINSON, M. J., 1995.** Inactivation of the bovine spongiform encephalopathy agent by rendering procedures. *Vet Rec* **137**, 605-10).
- TELLING GC, PARCHI P, DEARMOND SJ, CORTELLI P, MONTAGNA P, GABIZON R, MASTRIANNI J, LUGARESI R, GAMBETTI P, PRUSINER S.B., 1996.** Evidence for the conformation of the pathologic isoform of the prion protein enciphering and propagating prion diversity. *Science*, **274** 2079-2082.
- TELLING, G.C., SCOTT, M., MASTRIANNI, J., GABIZON, R., TORCHIA, M., COHEN, F.E., DE ARMOND, S.J., PRUSINER, S.B., 1995.** Prion propagation in mice expressing human and chimeric PrP transgenes implicates the interaction of cellular PrP with another protein. *Cell* **83**, 79-90.
- WELLS, G.A.H., HAWKINS, S.A.C., GREEN, R.B., AUSTIN, A.R., DEXTER, I., SPENCER, Y.I., CHAPLIN, M.J., STACK, M.J., DAWSON, M., 1998.** Preliminary observations on the pathogenesis of experimental bovine spongiform encephalopathy (BSE): an update. *Veterinary Record*, **142**, 103-106.
- WELLS, G.A.H., HAWKINS, S.A.C., GREEN, R.B., SPENCER, Y.I., DEXTER, I., DAWSON, M. 1999.** Limited detection of sternal bone marrow infectivity in the clinical phase of experimental bovine spongiform encephalopathy (BSE). *Veterinary Record*, **144**, 292-294.
- WILL, R. G., COUSENS, S. N., FARRINGTON, C. P., SMITH, P. G., KNIGHT, R. S. G., IRONSIDE, J.W., 1999.** Deaths from variant Creutzfeldt-Jakob disease. *Lancet* **353**, 979.

WOPFNER F., WEIDENHOFER G., SCHNEIDER R., VON BRUN A., GILCH S., SCHWARZ T.F., WERNER T., SCHUTZ HM., 1999. Analysis of 27 mammalian and 9 avian PrPs reveals high conservation of flexible regions of the prion protein. *Journal of Molecular Biology*, **289**, 1163-1178.

ZANUSSO, G., NARDELLI, E., ROSATI, A., FABRIZI, G-M., FERRARI, S., CARTERI, A., De SIMONE, F., RIZZUTO, N., MONACO, S., 1998. Simultaneous occurrence of spongiform encephalopathy in a man and his cat in Italy. *Lancet*, **352**, 1116-1117.

Annex 1:

BSE AGENT: NATURAL AND <u>EXPERIMENTAL</u> HOST RANGE					
PRIMATES	RUMINANTIA	FELIDAE	MUSTELIDAE	RODENTIA	OTHER ARTIODACTYLA
Man	Cattle	Domestic	<u>Mink</u>	<u>Mice</u>	<u>Pigs</u>
Lemur?	Nyala	cat			
Rhesus Monkey?	Gemsbok	Puma			
<u>Monkeys:</u>	Greater kudu	Cheetah			
<u>Marmoset</u>	Arabian	Ocelot			
<u>Macaque</u>	oryx	Tiger			
<u>Squirrel*</u>	Eland	Lion			
<u>Capuchin*</u>	Scimitar-				
<u>Lemurs?</u>	horned oryx				
	Ankole				
	<u>Bison bison</u>				
	<u>Sheep</u>				
	<u>Cattle</u>				
	<u>Goats</u>				

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Notes:

Hamsters and chickens challenged by the i/c route did not succumb

Chickens challenged by the oral route did not succumb.

Pigs challenged by the oral route did not succumb.

Annex 2: An example of a risk assessment of BSE transmission (Diringer, 1999):

Assay for BSE infectivity in the "mouse test" (across a species barrier)

Performance: 20 µl of a tissue suspension containing 2 mg of tissue are injected i.c. into groups of about 10-12 mice (total tissue tested: about 20 mg) (ref. 1)

Limit of detection: > 10 i.c. infectious doses per gram (probability of missing 10 i.c. doses per gram is 82%).

Risk assessment for transmission of BSE across a species barrier

Data for BSE: 10⁸ mouse i.c. infectious doses/kg of brain or spinal cord (CNS tissue) (CNS-tissue) (ref.1)
Ratio of efficiency: oral versus i.c. infection =1: 10⁵ (e.g., ref. 2)

Result for CNS-tissue: 1 kilo of CNS-tissue of an infected cow can contain 10³ mouse oral ID₅₀ for a susceptible species (in accordance with results of ref. 3, that applies to sheep and goats)

Limit of detection: > 10 i.c. infectious doses per g (see above)
equivalent to: 10⁴ i.c. infectious doses per kg
equivalent to: 0.1 oral infectious doses per kg

Result for any tissue tested for BSE-infectivity in the mouse:

1 kg of any cattle tissue negative for infectivity in the mouse test could contain 0.1 oral infectious dose for a susceptible species other than cattle. If ingested raw, regular consumption of such a dose could result in fatal disease in more than 10% of individuals. (Réf. 2).

Assays for infectivity in cattle (no species barrier)

Testing at the lowest dilution, altogether 400 mg of tissues in 4 cows (20 times more than in the mouse test) an increased sensitivity of at least 1000 times has been reported (ref. 4). Hence, one could expect concentrations of lethal BSE-infectivity for cattle at least 50 fold higher in the tissues.

Result for CNS-tissue: 1 kg of CNS-tissue of an infected cow at the clinical stage could contain up to 10⁵ oral infectious doses for cattle.

Result for other tissues: 1 kg of any tissue negative for infectivity in the mouse test could contain up to 10 oral infectious doses for cattle.

References:

1. Fraser H et al. *J. gen Virol* (1992) 73: 1891-1897
 2. Diringer H et al. *J gen Virol* (1998) 79: 606-612
 - 3 Foster JD et al. *Vet Rec* (1996) 138: 546-548
 - 4 Wells GAH et al. *Vet Rec* (1998) 142: 103-106
- See also Kimberlin, 1979 and Kimberlin and Walker 1989,1990.

Annex 3:

2. BIOLOGICAL STRAIN TYPING OF ISOLATES FROM SPECIES AFFECTED WITH TSE

By R.Bradley

1. INTRODUCTION

It is essential for epidemiological tracing and for the assessment of risk that agents causing TSE can be identified with precision and that any biological variation is distinguishable. In other words, isolates should be identified as a 'strain type'. The nature of TSE agents is still in dispute but there is clear evidence that the agent carries some form of informational molecule that determines the phenotype of disease in a given host. The following paragraphs examine how isolates from individuals affected with TSE can be identified and distinguished using in-bred strains of mice. The pattern of disease produced in regard to the incubation periods and microscopic lesion profiles (distribution and severity of vacuolation) in the brain is used to differentiate different strains. Since it is the reaction between the agent and the host (the mouse in this case) that is responsible for the biological variation, it is important to appreciate the following points:

- To note the difference between agent strain and mouse strain.
- Not all isolates transmit to mice. In this situation, the method cannot distinguish the causal strain.
- Isolates that do not transmit to mice should not be assumed to be of the same strain type.
- Some isolates may contain more than one strain.
- The isolate must be collected in a manner to exclude all possibility of cross-contamination with strains from other sources.
- The laboratory technique must be exemplary to eliminate the risk of subsequent cross-contamination.
- The titre of infectivity will affect the duration of the incubation period but not the lesion profile.
- Only the mouse strains mentioned here should be used as it cannot be guaranteed that other mouse strains will respond in the same way unless the mouse strain is validated against the base strains.
- If the incubation period is excessively long and approaching the lifespan of the particular mouse strain, lesions can occur spontaneously in the CNS as a result of ageing. It may be difficult to distinguish such changes from genuine pathology resultant from infection with the strain in question.
- During the strain typing process it may be necessary to clone the agent, that is to say to ensure that it is pure and comprises only one agent strain.
- Cloning is achieved by multiple passage in mice using a minimum infecting dose (in the region of 10^{-7} dilution).
- About 20 phenotypically distinct strains of scrapie and BSE have been isolated by serial passage in mice.
- It may be necessary to carry out second or third subpassages in mice before a clear scrapie strain type emerges.

3. 2. MOUSE STRAINS USED FOR GENOTYPING

Mice used for biological strain typing were developed before *PrP* genotyping was available. The mouse Scrapie incubation period gene, *Sinc*, is a major determinant of the length of the incubation period of different strains of scrapie and related agents. This gene is carried by all strains of mice other than those specially generated by gene deletion, so-called *PrP* knockout mice. There are two alleles *s7* and *p7*. The allele 's' stands for short and 'p' for prolonged incubation period, following inoculation with the ME7 strain of scrapie. The *Sinc* gene is now regarded as coincident with the *PrP* gene *prn-p* that has two alleles *prn-p^a* corresponding to *s7* and *prn-p^b* corresponding to *p7*. The polymorphisms responsible for the differences between alleles have been described. The amino acid sequence of the protein PrP that is encoded by the two different alleles consistently differs by two amino acids. In the ensuing discussion, the term *Sinc* will be used because most of the scientific papers dealing with strain typing use this terminology. (Hunter et al, 1992; Moore et al, 1998).

Conventional mice are *Sinc s7s7* or *Sinc p7 p7*. Though there are several strains of mice of each type, in practice only four are used for strain typing in the context of attempting to distinguish the BSE agent from scrapie agents. These are RIII and C57 black mice which are both *Sinc s7s7*, and VM and IM mice which are both *Sinc p7p7*. Also used is the F₁ hybrid C57 black x VM which is *Sinc s7p7*.

When scrapie agent strain ME7 is inoculated into *Sinc s7s7* mice the incubation period is significantly shorter than following inoculation of *Sinc p7p7* mice. The incubation period in the F₁ hybrid lies between the two. Strains exhibiting this pattern (but with different incubation periods and lesion profiles) are of the 'ME7 type'. By contrast, inoculation of scrapie strain 22A results in precisely the reverse, namely a shorter incubation in *Sinc p7p7* mice than in *s7s7* mice. Furthermore, there is an over dominant effect in the F₁ hybrid in that the incubation period is now longer than in either of the other homozygotes. Strains that operate in this way are of the '22A type'.

4. 3. DIFFERENTIATION OF STRAINS OF TSE AGENT

Strains are distinguished by measurement of the incubation period and assessment of the lesion profile in specified in-bred strains of mice.

The incubation period is determined by measuring the interval in days between, i/c inoculation of 20 microlitres of a 10% brain homogenate from the source animal, and a standard clinical endpoint. Around 20 mice of each type may be inoculated. The clinical endpoint is when any individual mouse is scored as being definitely affected following daily observation, using strict clinical criteria. A mean incubation period is calculated for any particular mouse strain.

The lesion profile is measured in nine grey matter and three white matter areas of the brain. Vacuolation is scored subjectively on the basis of lesion severity on scales of 1–5 and 1–3 respectively. The lesion profiles can be presented in graphical form with the mean lesion score from all surviving mice in the group on the vertical axis and the numbered brain region on the horizontal axis. This allows visual comparison of the profile. Strains with similar profiles can be easily recognised and distinguished from strains with different profiles. Being a biological system there is a degree of variation between the profiles observed in different mice of the same strain, but this variation is generally small. The differences in the

profile produced by different strains of agent in the same mouse strain are significant and usually are readily distinguished.

The position of the F₁ hybrid in regard to incubation period length may also help to distinguish the strain.

In the case of the BSE agent strain, no matter what species it is derived from at primary passage, it shows a consistent incubation period difference in the two strains of mice with the same *Sinc* genotype. This is a useful distinguishing feature of this strain at primary (*e.g.* cow to mouse) passage. At second and subsequent passages, this feature is lost.

To certainly determine all the strains present in an isolate (there may be one or more than one) and to precisely identify them, it is necessary to sub-passage the agent in the same strain of mouse or possibly in the alternative homozygote. This procedure is somewhat complex. As an example, in regard to the BSE agent when passaged in C57 black or VM mice, new strains called 301C and 301V are isolated. These are different from any strains isolated so far from sheep with scrapie.

With the development of immunohistochemical staining for PrP another aid to strain, differentiation has been established. This is because the distribution of PrP-related pathology is dependent upon the strain of agent. Different populations of neurons are targeted by different strains but there is also some overlap. Thus, some neuron populations respond pathologically to infection with different strains of agent. Some strains of agent target with great precision whilst others produce a more diffuse reaction.

Thus, there are collectively a number of pointers that readily distinguish, on a repeatable basis, the BSE strain from other strains. A strain with the same characteristics as the BSE agent has been isolated from three cats with FSE, a greater kudu and a nyala with a TSE and from a pig, sheep and goat with experimental BSE. A strain with indistinguishable characteristics has also been isolated from the brains of three patients with vCJD. Strains isolated from three brains from patients with sporadic CJD are distinguishable from the BSE agent strain.

5. 4. STRAINS OF MOUSE-ADAPTED SCRAPIE AGENTS

Following primary transmission of field isolates to mice, the incubation period is generally long and variable due to the donor species effect. This has been attributed to the difference in sequence of the PrP between the donor and recipient species but it is not possible to predict from donor PrP genotype the incubation period at first passage in mice. At secondary passage, the incubation period is often shorter and this phenomenon indicates the presence of a species barrier. Following further passages the incubation period usually stabilises. If the primary isolations are done in the two different mouse homozygotes different strains may sometimes, but not always, be isolated.

Some strains such as ME7, 22A, 22C, 79A, 87V and 139A are extremely stable following multiple passage. However, other strains such as 87A are only stable under particular conditions of passage. At other times, such as after passage at high dose in a particular genotype a strain (in this case a strain indistinguishable from ME7) with a much shorter incubation period is isolated which is itself stable. The explanation for this is that isolates of

87A probably contain very low concentrations of ME7 that are only revealed after passage at high dose. It is presumed that ME7 is derived from 87A by a process akin to mutation.

Some strains like cloned ME7 and cloned 22C retain their strain characteristics after passage in the alternative *Sinc* homozygote whereas others do not. This also follows when cloned ME7 or 22A are passaged through hamsters and then back to mice. Other strains such as cloned 22C and cloned 139A result in the isolation of different strains. This result is not consistent if the species is changed. For example when cloned strain 139A is passed through rats and re-isolated in mice the strain is unchanged. Three alternative explanations have been offered for these observations:

- Despite cloning a minor strain is present
- A mutation-like event has occurred and the new strain is selected
- The host has effected the change in an unknown way

Particular mouse and agent strain combinations are used experimentally because of their special properties. For example 301V in mouse and 263K in hamster can be grown to high titre and are useful for spiking experiments in inactivation studies. The 22A strain is heat resistant and is similarly useful for this purpose.

Interestingly the 263K strain was derived originally from a mouse strain but has very low pathogenicity for the mouse despite the fact that it can be grown to high titres in hamsters. This strain was used to first isolate PrP and thus lay the foundation for the prion hypothesis.

Strain 87V in *Sinc* p7p7 mice when inoculated i/p does not neuro-invade so no disease results despite high titres of infection in the spleen. If inoculated i/c disease results in conventional fashion. Thus particular agent/mouse strain models are used a great deal in research to investigate phenomena of pathogenesis, inactivation, mutation and other features of these fascinating experimental diseases.

EXPERIMENTS USING PASSAGED ISOLATES IN ANOTHER SPECIES

It is important to note that the strain of agent in an original isolate may or may not exist in brain pools derived from subsequent passage across a species barrier. For example, pooling primate brains for further transmission studies following primary transmission of a human TSE, when used in further experiments may not reflect what would happen with a direct human to human passage. This of course cannot be done. Caution should be exercised in interpreting the results of such studies. This is because firstly, the primates may be genetically diverse. Secondly, the original human-derived agent may be lost, altered, or may be present in very low concentration because other strains have been preferentially selected.

SHEEP SCRAPIE BRAIN POOLS

For experimental studies in sheep, it is necessary to have a consistent challenge inoculum that will produce consistent and predictable outcomes in particular breeds and genotypes of sheep. Two main pools have been used in experimental studies. These consist of either A type strains or C type strains differentiated in terms of the PrP genotypes of the sheep which develop scrapie following experimental challenge. The first is Sheep Scrapie Brain Pool 1 (SSBP/1) that has been repeatedly passaged in Cheviot sheep. This pool, consisting predominantly of A type strains, produces a consistent phenotype of scrapie in particular *PrP* genotypes. One feature of the disease produced is the minimal neuropathology but

characteristic presence of PrP^{Sc}. Some genotypes are refractory to subcutaneous inoculation with this pool. Some of these resistant sheep are however, susceptible if the pool is inoculated i/c. Sheep with different *PrP* genotypes respond with different incubation periods.

CH1641 is also a mixture of strains but this time predominantly of C type. CH1641 has also been repeated passaged in Cheviot sheep and this inoculum produces disease in sheep of different genotype to those which are susceptible to SSBP/1.

There are similarities in certain aspects of transmission (susceptible PrP genotypes) and PrP protein derived from Cheviot sheep inoculated with CH1641 and BSE agent from cattle. However, the agents are clearly distinguishable because BSE agent readily and consistently transmits to all five mice types mentioned above whereas CH1641 has a low pathogenicity for mice.

For further information on biological strain typing see Fraser and Dickinson, (1968), Bruce *et al*, (1989), Bruce, (1993, 1996).

6. 5. REFERENCES

- BRUCE, M.E., (1993).** Scrapie strain variation and mutation. *British Medical Journal*, **49**, 822-838.
- BRUCE, M.E., (1996).** Strain typing studies of scrapie and BSE. In *Methods in molecular medicine: Prion diseases*. H. Baker, R.M Ridley eds. Human Press, Inc. Totowa, NJ. Pp 223-236.
- BRUCE M.E., McBRIDE, P.A., FARQUHAR C.F., (1989).** Precise targeting of the pathology of the sialoglycoprotein, PrP, and vacuolar degeneration in mouse scrapie. *Neuroscience Letters*, **102**, 1-6.
- FOSTER J.D., DICKINSON A.G., (1988).** The unusual properties of CH1641, a sheep passaged isolate of scrapie. *Veterinary Record*, **123**, 5-8.
- FRASER H., (1993).** Diversity in the neuropathology of scrapie-like diseases in animals *British Medical Journal*, **49**, 792-809.
- FRASER H., DICKINSON A.G. (1968).** The sequential development of the brain lesions of scrapie in three strains of mice. *Journal of Comparative Pathology*, **78**, 301-311.
- HUNTER N, DANN JC, BENNETT AD, SOMMERVILLE RA, McCONNELL I, HOPE J, 1992.** Are Sinc and the PrP gene congruent? Evidence from PrP gene analysis in Sinc congenic mice. *Journal of General Virology* **73**: 2751-2755
- MOORE RC, HOPE J, McBRIDE PA, McCONNELL I, SELFRIDGE J, MELTON D, MANSON JC, 1998.** Mice with gene targetted prion protein alterations show that Prnp, Sinc and Prni are congruent. *Nature Genetics* **18**: 118-125]