RESULTS and DISCUSSION
of a PROCESS VALIDATION STUDY
of the REMOVAL of TSE (Scrapie) INFECTIVITY
DURING the MANUFACTURE of Regeneresen®

by

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RESULTS

1. Titration of the scrapie spike (brain homogenate)

The validation studies of Regeneresen® were carried out using the 263K strain of hamster-passaged scrapie agent. The scrapie spike consisted of a macerate of pooled brains taken at the late clinical stage of the disease.

The titre of the spike was determined by homogenising 110 g. of the macerated hamster brain in a total volume of 562ml. of solution. Therefore the homogenate contained 19.6 percent brain (weight / volume).

Table 1 shows the results of the titration in hamsters that were observed for the standard observation period of 360 days after intracerebral (i.c.) injection.

Table 1: Titration of the scrapie spike - ASSAY 3

Dilution of the spike	Number of hamsters that died, were killed or survived			$\frac{B}{B+C}$
	A. Died ‡	B. Scrapie *	C. Survivors (360 days)	
10 (-5)	0	10	0	1.0
10 (-6)	0	10	0	1.0
10 (-7)	0	10	0	1.0
10 (-8)	0	7	3	0.7
10 (-9)	2	0	8	0.0
10 (-10)	0	0	10	0.0
10 (-11)	0	0	10	0.0
Log (10) titre / 50 µl. of spike				8.2
Log (10) titre / ml. of spike				9.5
Arithmetic titre / ml. of spike =		3.2 x 10 (9)		

‡ Negative / * positive histology for scrapie

The titre of the 19.6% scrapie brain homogenate was 3.2×10^9 i.c. LD_{50} / ml. Multiplying this value by a factor of 5 gives a titre of 1.6×10^{10} i.c. LD_{50} / g. of scrapie-affected hamster brain. This is within the expected range from published studies (Kimberlin & Walker, 1989).

2. The size of the spike for ASSAYS 1 and 2

The validation study was carried out in duplicate on a scale that was 1/10th of the normal manufacturing process.

The starting materials in the two experiments consisted of 249.5g. and 250.1g. of scrapie-brain macerate, respectively, an average of 250g.

From the scrapie infectivity titre shown above, the total input titre was:-

$$250\text{g. X } 1.6 \times 10^{10} = \mathbf{4.0 \times 10^{12}\ i.c.\ LD_{50}}$$

3. Removal factor for the product before autoclaving: ASSAY 1

Duplicate experiments (A and B) were carried out separately, in series. In both experiments, about 20% of the bio-assay hamsters injected with the most concentrated (neat) sample developed scrapie. But, there were no cases of scrapie in hamsters injected with serial 10-fold dilutions of the samples after the standard observation period of 360 days (see Table 2 on the next page).

Table 2 shows that the arithmetic titre of the injected sample was 1.0×10^1 i.c. LD_{50} / ml.

However, three correction factors are needed to make the titre of the sample equivalent to the total input titre. This is necessary in order to calculate the reduction in scrapie infectivity during the manufacture of the product.

FIRST, the sample was diluted 1:2 before it could be injected into the bio-assay hamsters: a multiplication factor of 3.

Table 2: Titration of the product BEFORE autoclaving - ASSAY 1

Dilution of the sample	Number of hamsters that died, were killed or survived			$\frac{B}{B+C}$
	A. Died ‡	B. Scrapie *	C. Survivors (360 days)	
10 (0)	0	2	10	0.2
Duplicate	0	2	10	0.2
10 (-1)	1	0	11	0.0
Duplicate	1	0	11	0.0
10 (-2)	1	0	11	0.0
Duplicate	0	0	12	0.0
10 (-3)	2	0	10	0.0
Duplicate	1	0	11	0.0
10 (-4)	0	0	12	0.0
Duplicate	1	0	11	0.0
10 (-5)	0	0	12	0.0
Duplicate	1	0	11	0.0
Log (10) titre / 50 µl. of sample				-0.3
Log (10) titre / ml. of sample				1.0
Arithmetic titre / ml. of sample =		1.0 x 10 (1)		

‡ Negative / * positive histology for scrapie

SECONDLY, since the total volume of the undiluted sample was 100ml., a multiplication factor of 100 is needed.

THIRDLY, the average yield of brain RNA in the duplicate experiments was 105mg. (individual values were 103.2mg. and 107.1mg.). Since aliquots of about 25mg. (24.0mg. and 26.5mg., respectively) were used to formulate the brain RNA into the product, the multiplication factor is 4.2. This factor is based on the assumption that there was no loss of material during the subsequent centrifugation and filtration steps.

The combined multiplication factor is 3.0 X 100 X 4.2 = 1,260

Therefore, the EQUIVALENT amount of infectivity in the product before autoclaving was:-

$$1.0 \times 10^{1} \text{ X } 1{,}260 = \underline{\mathbf{1.3 \times 10^{4}\ i.c.\ LD_{50}}}$$

Since the input titre was 4.0×10^{12} i.c. LD_{50}, the total reduction of infectivity during the purification of the product was:-

$$\frac{4.0 \times 10^{12}}{1.3 \times 10^{4}} = 3.1 \times 10^{8} = \underline{\mathbf{310\ million\text{-}fold}}$$

4. Removal factor for the full manufacturing process: ASSAY 2

No infectivity was detected in either experiment A or B when hamsters were observed for 360 days after injection.

Extending the observation period to 450 days for the animals injected with the neat samples still did not result in the occurrence of scrapie cases (see Table 3 on the next page).

However, it is possible that there may have been some undetected infectivity in the samples. The maximum amount that could have been present can be calculated from the limits of detectability of the bio-assays.

Table 3 shows that the limit of detectability of the bio-assay was $\underline{6.3 \times 10^{0}\ \text{i.c.}\ LD_{50}}$ / ml.

The combined correction factor for the dilution of the samples (1:2), the volume of the undiluted sample (100ml.), and the proportion of the purified RNA that was formulated into the final product (multiplication factor of 4.2) was the same as before, that is, <u>1,260</u>

Therefore, the EQUIVALENT amount of undetected infectivity in the product after autoclaving was:-

$$6.3 \times 10^{0} \text{ X } 1{,}260 = \underline{\mathbf{7.9 \times 10^{3}\ i.c.\ LD_{50}}}$$

Table 3: Titration of the product AFTER autoclaving - ASSAY 2

Dilution of the sample	Number of hamsters that died, were killed or survived			B / B + C
	A. Died ‡	B. Scrapie *	C. Survivors (450 days)†	
10 (0)	7	0	24	0.0
Duplicate	8	0	23	0.0
10 (-1)	0	0	12	0.0
Duplicate	1	0	11	0.0
10 (-2)	1	0	11	0.0
Duplicate	0	0	12	0.0

Log (10) titre / 50 µl. of sample -0.5
Log (10) titre / ml. of sample 0.8
Arithmetic titre / ml. of sample = 6.3 x 10 (0)

‡ Negative / * positive histology for scrapie
† Observation period was 360 days for the two lower dilutions.

Since the input titre was 4.0×10^{12} i.c. LD_{50}, the total reduction of infectivity during the purification and terminal autoclaving of the product was:-

$$\frac{4.0 \times 10^{12}}{7.9 \times 10^{3}} = 5.1 \times 10^{8} = \underline{\textbf{510 million-fold}}$$

This value should be regarded as the minimum reduction factor for the full manufacturing process (see DISCUSSION).

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DISCUSSION

The significance of these results is discussed in relation to several important features in the design of the study.

1. Choice of the model for the BSE agent and observation periods

It is generally accepted that experiments with the well characterised models of rodent scrapie are suitable for investigating the removal and inactivation of any of the transmissible spongiform encephalopathy (TSE) agents during the manufacture of medicinal products from human or animal tissues (see Section 3.6 in EC, 1991).

The present studies were carried out with the 263K strain of scrapie which had been biologically cloned in hamsters (Kimberlin & Walker, 1977, 1978). This model of scrapie offers two advantages over other well characterised models that are currently available.

FIRST, the brains of clinically-affected hamsters contain exceptionally high titres of infectivity. This makes it possible to demonstrate relatively high removal factors.

SECONDLY, the incubation periods for limiting infectious doses of the 263K agent are not too close to the life-span of the host. This is important in minimising the risk of obtaining falsely negative results if too many injected hamsters die from causes unrelated to scrapie. Tables 1-3 show that this did not happen.

At the same time, decisions about exactly how long hamsters should be observed are subject to the law of diminishing returns; that is, the progressively increased costs and reduced value of the extra information obtained by prolonging observations beyond certain biologically defined limits.

In the present studies, these limits were defined in relation to the incubation periods at limiting infectious doses of the 263K scrapie agent in intracerebrally-injected hamsters.

Published studies on 10 different scrapie-affected brain pools indicate an average incubation period plus 3 standard deviations of about 175 days for injected doses of 1-10 i.c. LD_{50} units (Kimberlin & Walker, 1989).

The standard observation period of 360 days was derived by increasing this value 2-fold to allow for the occurrence of long-incubation cases of scrapie, as often happens at average doses of 1 i.c. LD_{50} unit, or less. The extended observation period of 450 days that was used for the neat samples in ASSAY 2 was based on a multiple of about 2.5-times 175 days.

In the present study, the longest incubation period in the bio-assay of the scrapie spike (ASSAY 3) was 222 days. This is well within the designated observation time of 360 days.

The 4 hamsters that developed scrapie in ASSAY 1 had incubation periods of 153 days, 193 days, 220 days and 313 days, respectively. Even the longest incubation period (313 days) was within the 360-day observation period and it was about 140 days less than the observation time of 450 days that was used in ASSAY 2 for the neat samples when no cases of scrapie occurred.

Nevertheless, it cannot be proved that rare cases of scrapie with extremely long incubation periods might not have occurred had the hamsters been observed for even longer times.

The consequences of this happening can be calculated by considering a most unlikely situation; namely, that 50% of all the hamsters that survived 450 days after injection with the neat samples in ASSAY 2 were in fact incipient cases of scrapie.

Table 4: Theoretical consequences if 50% incipient scrapie cases had occurred in hamsters injected with neat inoculum in ASSAY 2.

	Removal Factors		
	Actual	Theoretical	Difference
ASSAY 2	510 million	160 million	3.2-fold lower

It is obvious from Table 4 that even this highly improbable scenario would not alter the reduction factors substantially; slightly more than 3-fold.

2. Factors influencing the design and interpretation of the study

Manufacturing Process

The design of the validation study was based on an evaluation of the manufacturing process in relation to the known physico-chemical properties of the TSE agents.

Despite continuing uncertainties about the precise chemical nature of the TSE agents, there is no doubt that protein is an essential constituent (Cho, 1980, 1983; Lax et al., 1983; McKinley et al., 1983; Marsh & Hanson, 1969; Millson et al., 1976; Prusiner et al., 1981).

The active ingredient of Regeneresen® is low molecular weight RNA which is purified by simple classical techniques. These involve the use of a strong detergent (sodium dodecyl sulphate) which, together with several precipitation and centrifugation steps, removes protein and, therefore, would be expected to remove a substantial amount of TSE infectivity. The study confirmed this expectation (see Results of ASSAY 1).

It was also expected that any remaining infectivity would be inactivated when the purified product was sterilised in sealed ampoules. Because of the high physico-chemical stability of nucleic acids to heat, terminal sterilisation is carried out under very stringent conditions; namely, autoclaving at 134° C for 18 minutes. These conditions are known to provide one of the most effective ways of inactivating TSE agents (Kimberlin et al., 1983: Brown et al., 1986; Taylor et al., 1994).

It is difficult to imagine that any TSE contamination of the purified product in solution would survive terminal sterilisation under these conditions. That is why the primary objective of the validation experiments was to test the ability of the whole purification process to remove extremely high levels of scrapie infectivity, before autoclaving (ASSAY 1).

However, material was also bio-assayed after the autoclave step (ASSAY 2) in order to provide a total reduction factor for the whole manufacturing process.

Study Design

A practical difficulty with TSE validation studies is that infectivity has to be added, not as purified agent, but as a crude brain homogenate. Therefore, infectivity will behave heterogeneously depending on the size of the various tissue components with which it is associated, and the effects of processing on them.

This does not matter when scrapie-affected brain is added to the homogenised source tissue from which the product is derived but it can have serious consequences if infectivity is added to test the potential of later (downstream) stages of the manufacturing process to remove or inactivate agent.

The reason is that infectivity in a downstream spike has not been through the earlier processing stages and the sub-population of infectious agent that is most easily inactivated or removed from the initial spike will be so again from the downstream spike.

This is especially true of physical separation methods (see Section 5.2 in EC, 1991) and the consequence can be to exaggerate the reduction factor for the whole process obtained by multiplying the results from several spikes.

In the present study, this problem was **completely avoided** by adding scrapie infectivity only at the beginning of the manufacturing process. Two exceptional features in the manufacture of Regeneresen® made this possible.

FIRST, Regeneresen® is manufactured from relatively small batches of tissue (about 2.5 kg.) so that the study could be carried out using a 1/10th facsimile of the normal production process. This, and the fact that the process involves chemical extractions, low-speed centrifugations and simple filtrations, made for a very accurate facsimile.

SECONDLY, since brain is the source tissue for many preparations of Regeneresen®, and because of the small scale of the normal production process, it was possible to use scrapie-affected hamster brain as the **sole** starting material for the validation study.

This is in complete contrast to the usual practice when the scrapie spike necessarily constitutes only a small proportion of other types of starting tissue.

The fact that the validation experiments also used hamster brain infected with very high titres of the 263K strain of agent **greatly increased the maximum reduction factor that could be demonstrated for the whole production process without creating any difficulties of interpretation.**

3. Significance of the results

Results

The most significant finding of the study was that the reduction of scrapie infectivity during the production of Regeneresen® was 310 million-fold, BEFORE the terminal sterilisation step.

However, a consequence of this was that it was not possible to demonstrate an additional reduction factor, AFTER autoclaving at 134°C for 20 minutes, that was greater than about 1.6-fold.

In reality, a reduction of several orders of magnitude would be expected from the results of other studies (Kimberlin et al., 1983; Brown et al., 1986; Taylor et al., 1994). For example, when an exceptionally heat-stable strain of scrapie agent was autoclaved at 136°C for 4 minutes, no infectivity was detected thus giving a minimum reduction factor of 5.6 $\log_{10}$ units; that is, 400,000-fold (Kimberlin et al., 1983).

If, for the purpose of illustration, terminal autoclaving had reduced the infectivity by only 0.008% of the above value, that is 32-fold, then the total reduction factor for the production of Regeneresen® would have been:-

32 x 310 million = 10,000 million.

Therefore, the reduction factor of 510 million obtained for the whole manufacturing process of Regeneresen® represents a substantial underestimate of the true value.

Significance

The significance of the reduction factor actually obtained can be illustrated by incorporating it into a "worst-case" assessment of the risk to patients from Regeneresen® made from foetal or neonatal bovine brain.

Regeneresen® is an intrinsically low risk product for two reasons.

FIRST, the brain is sourced exclusively from Germany, a low-risk country with respect to BSE (Kimberlin, 1996).

SECONDLY, the source animals are of an age that is well before the time when the infectious agent has reached the brain (Kimberlin & Walker, 1988; Wells et al., 1996). In this situation, the "worst-case" assumption is that infectivity is present in an amount equivalent to the limit of detectability of the bio-assay, that is 3.2 i.c. LD_{50} cattle units / g. brain (Kimberlin, 1996). This is **1,000 million times lower** than the scrapie titre of the spike in hamsters (Table 1).

A very simple calculation of risk can be made according to published principles using only 5 pieces of information (Kimberlin, 1996).

(A) IF the weight (g.) of brain used in a maximum treatment regime with Regeneresen® $= 1.2 \times 10^4$

(B) IF the prevalence of BSE infection in the source animals $= 1.0 \times 10^{-5}$

(C) IF the BSE titre in foetal & neonatal brain (limit of detectability: i.c. LD_{50} / g.) $= 3.2 \times 10^0$

(D) IF the relative efficiency of BSE infection by the i.m. route of administration of Regeneresen® compared to the i.c. route $= 1.0 \times 10^{-2}$

(E) IF 1 / the BSE reduction factor during the manufacture of Regeneresen® $= 2.0 \times 10^{-9}$

THEN the maximum exposure of 1 patient to BSE infectivity is A x B x C x D x E which is **7.7×10^{-12} LD_{50}** units.

This means that there is a 50:50 chance of 1 patient in 130,000 million receiving a lethal dose of BSE infectivity; that is, a risk of **1 in 260,000 million** patients.

Even this extremely low risk assumes that terminal autoclaving of Regeneresen® reduces BSE infectivity by less than a factor of 2. There are also 3 other severe "worst-case" assumptions:-

(a) that 1 LD_{50} unit is physically indivisible and would always be associated with a 50% chance of causing infection;

(b) that infection would always lead to disease;

(c) and that there is no species barrier, whatsoever, between cattle and man.

It is instructive to put this estimated risk to patients treated with Regeneresen® in the context of the risk of Creutzfeldt-Jakob disease (CJD) in the general human population.

The incidence of non-iatrogenic, sporadic CJD is remarkably constant throughout the world at approximately 1 case per million people per year (Brown et al., 1987; Will et al., 1986). Therefore, the lifetime risk of CJD to any one individual is about 1 in 10,000 (Brown et al., 1985).

It is concluded that the "worst-case" estimate of the risk of spongiform encephalopathy due to Regeneresen® is at least 10 million times lower than the risk of sporadic CJD.

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Scientific Study Monitor

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REFERENCES

Brown, P., Gajdusek, D.C., Gibbs, C.J. jun. & Asher, D.M. (1985) Potential epidemic of Creutzfeldt-Jakob disease from human growth hormone therapy. New England Journal of Medicine 313, 728-731.

Brown, P., Rohwer, R.G. & Gajdusek, D.C. (1986) Newer data on the inactivation of scrapie virus or Creutzfeldt-Jakob disease virus in brain tissue. Journal of Infectious Diseases 153, 1145-1148.

Brown, P., Cathala, F., Raubertas, R.F., Gajdusek, D.C. & Castaigne, P. (1987) The epidemiology of Creutzfeldt-Jakob disease: Conclusion of a 15-year investigation in France and review of the world literature. Neurology 37, 895-904.

Cho, H.J. (1980) Requirement of a protein component for scrapie infectivity. Intervirology 14, 213-216.

Cho, H.J. (1983) Inactivation of the scrapie agent by pronase. Canadian Journal of Comparative Medicine 47, 494-496.

EC (1991) European Commission, Committee for Proprietary Medicinal Products (1991) Notes for guidance on validation of virus removal and inactivation procedures. Document No. 111 8115/89.

Kimberlin, R.H. & Walker, C.A. (1977) Characteristics of a short incubation model of scrapie in the golden hamster. Journal of General Virology 34, 295-304.

Kimberlin, R.H. & Walker, C.A. (1978) Evidence that the transmission of one source of scrapie agent to hamsters involves separation of agent strains from a mixture. Journal of General Virology 39, 487-496.

Kimberlin, R.H., Walker, C.A., Millson, G.C., Taylor, D.M., Robertson, P.A., Tomlinson, A.H. & Dickinson, A.G. (1983) Disinfection studies with two strains of mouse passaged scrapie agent: guidelines for Creutzfeldt-Jakob and related disease. Journal of the Neurological Sciences, 59, 355-369.

Kimberlin, R.H. & Walker C.A. (1988) Incubation periods in six models of intraperitoneally injected scrapie depend mainly on the dynamics of agent replication within the nervous system and not the lymphoreticular system. Journal of General Virology, 69, 2953-2960.

Kimberlin, R.H. & Walker, C.A. (1989) Transport, targeting and replication of scrapie in the CNS. In: Unconventional Virus Diseases of the Central Nervous System. Edited by L. Court, D. Dormont, P. Brown and D. Kingsbury, pp. 547-562. Commissariat a l'Energie Atomique, Fontenay-aux-Roses.

Kimberlin, R.H. (1996) Bovine spongiform encephalopathy (BSE) and public health: some problems and solutions in assessing the risks. In: Transmissible Subacute Spongiform Encephalopathies: Prion Diseases. Edited by Louis Court and Betty Dodet, pp. 487-502. Elsevier, Paris.

Lax, A.J., Millson, G.C. & Manning, E.J. (1983) Involvement of protein in scrapie agent infectivity. Research in Veterinary Science 34, 155-158.

McKinley, M.P., Bolton, D.C. & Prusiner, S.B. (1983) A protease-resistant protein is a structural component of the scrapie prion. Cell 35, 57-62.

Marsh, R.F. & Hanson, R.P. (1969) Physical and chemical properties of the transmissible mink encephalopathy agent. Journal of Virology 3, 176-180.

Millson, G.C., Hunter, G.D. & Kimberlin, R.H. (1976) The physicochemical nature of the scrapie agent. In: Slow Virus Diseases of Animals and Man. Edited by R.H. Kimberlin, ch. 11, pp. 243-266. North-Holland, Amsterdam.

Prusiner, S.B., McKinley, M.P., Groth, D.F., Bowman, K.A., Mock, N.I., Cochran, S.P. & Masiarz, F.R. (1981) Scrapie agent contains a hydrophobic protein. Proceedings of the National Academy of Science, Washington 78, 6675-6679.

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.

Wells G.A.H., Dawson ,M., Hawkins, S.A.C., Austin, A.R., Green, R.B., Dexter, I., Horigan, M.W., Simmons, M.M. (1996) Preliminary observations on the pathogenesis of experimental bovine spongiform encephalopathy. In: Bovine Spongiform Encephalopathy: The BSE Dilemma. Edited by C.J.Gibbs, jun. pp 28-44. Springer-Verlag, New York.

Will, R.G., Matthews, W.B., Smith, P.G. & Hudson, C. (1986) A retrospective study of Creutzfeldt-Jakob disease in England and Wales 1970-1979 II: epidemiology. Journal of Neurology, Neurosurgery and Psychiatry 49, 749-755.

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