REMOVAL OF CAUSATIVE AGENT OF CREUTZFELDT-JAKOB DISEASE (CJD) THROUGH MEMBRANE FILTRATION METHOD

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クロイツフェルトヤコブ病 (CJD) の原因物質の膜除去方法

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We intended to show the possibility of the removal of the Creutzfeldt-Jakob disease (CJD) causative agent by the filtration using the virus removal membrane BMM®, that is, cuprammonium regenerated cellulose hollow fiber. The BMMs with various mean pore size $2\bar{r}_f$ ranging between 35 and 75 nm were employed. The supernatant of the centrifuged homogenate obtained from the mouse brains infected with CJD was filtered through BMMs and each filtrate was injected intracerebrally into mice. The concentration of the CJD infectious agent was evaluated through the mean death day caused by CJD after the injection. The dependence of this concentration of the filtrate on $2\bar{r}_f$, the change in this concentration of the double filtration, and the change in the concentration of the filtrate from the solution after the treatment of the surfactant "Sarkosyl" was investigated. The infection of CJD was judged from the pathology of spongiform encephalopathy and from the generation of the CJD isoform of Prion Protein (PrPCJD).

The results were summerized as follows : (1) The infectivity of the filtrate decreased when $2\bar{r}_f$ decreases, and after the filtration using the BMM with $2\bar{r}_f$ value of 35 nm, this infectivity disappeared completely indicating the logarithmic rejection coefficient was more than 6. (2) The double step filtration using two BMMs with $2\bar{r}_f$ of 60 nm gave about 10% increase in the rejection coefficient comparing with the case of the single step filtration. (3) The treatment of the Sarkosyl made the permeability of the CJD causative agent through the BMM with $2\bar{r}_f$ of 40 nm increased to 100% and the infectivity increased to about five hundreds times of that of the original solution.

Key words : BMM®, Creutzfeldt-Jakob Disease, Pathogen Removal, Filtration, BSE (Bovine Spongiform Encephalopathy), Prion Protein, Inactivation

INTRODUCTION

The progress in a bio-technology accelerates the development of valuable bio-drugs. In order to minimize the probability of infections by microbes, the guidelines or guidances have been completed in many countries¹⁻⁵⁾. The unknown infectious agents such as Creutzfeldt-Jakob disease (CJD), Scrapie, and Bovine Spongiform Encephalopathy (BSE) become new target pathogens to be removed. All of these are regarded as a kind of the Prions and are recognized in an animal body fluid which is employed in a culture medium or in human body fluid employed in bio-drugs. In order to protect the infection of BSE to human beings from bio-drugs, the guidance of the title of "Guidelines for minimizing the Risk of Transmitting Agents Causing Spongiform Encephalopathy via Medical Products" has been published recently⁶⁾. In the case of CJD, the removal of this pathogen has been demanded from regulation authorities.

Although the characteristics of the prions, small proteinaceous infectious particles have not been completely known yet, the vigorous resistance against the inactivation by the procedures which modify nucleic acid is common feature. The prions can be distinguished from viruses through the following points of the multiple molecular forms, the non-immunogenic response, no evidence for a proper nucleic acid, and the protein component encoded by a chromosomal gene. Because of the above characteristics of the prions, there are several barriers to the investigation of technologies to prevent its infections. For example, the assay method to detect the infectivity is limited in only *in-vivo* experiments and the window period of the infection is more than two year. The possibility to remove the prions using polymeric membrane with 25 nm in mean pore size has been proved by Tateishi *et al*⁷⁾, and except this study no study has been succeeded so far.

On the other hand, the virus removal membrane,

named as BMM (cuprammonium regenerated cellulose hollow fiber), has been known to be able to remove particles mainly based on their size. The particle size can be determined through the change in the infectivity with $2\bar{r}_f$ using this membrane⁸⁾. Accordingly, when we know the change in the CJD infectivity of a filtrate as the function of $2\bar{r}_f$, the size of CJD in the actual existence condition may be determined.

In this study, we intend to show the removability of CJD using the virus removal membrane as a function of membrane characteristics through the dependence of the infectivity on mean pore size $2\bar{r}_f$ and the change in the infectivity with a double step filtration and the effect of a surfactant on the infectivity of a filtrate.

EXPERIMENT

Sample Preparation

(a) CJD infectious solution : The initial infectious homogenate was prepared through the following procedure ; The 3 g of brains of mice⁷⁾ infected with CJD was homogenized into 40 ml of distilled water and centrifuged under the condition of 3000 rpm at 20°C for 30 min. The supernatant thus obtained was diluted into 290 ml using saline water and then freeze-dried into -60°C. The solution was remelted at 40°C and then was centrifuged under the condition of 2000 rpm at 20°C for 10 min and the supernatant was prepared just before filtration.

Ten fold dilution of the initial homogenate was prepared to get the calibration curve of the death day after intracerebral inoculation vs. dilution ratio⁷⁾.

(b) Virus removal membrane ; The series of BMM[®]s with different mean pore size ranging between 35 nm and 75 nm were provided from Asahi Chemical Ind. Co. Ltd. The filtration area was 0.7 cm².

(c) Animals ; Weanling female mice (NZW strain) were obtained from closed colonies in the

Institute for Animal Experimentation, Kyushu University, and kept in specific-pathogen-free (SPF) conditions throughout the experiment.

Measurement :

(a) Assay of CJD ; Injection of 0.02 ml of the sample solution was given intracerebrally into a mouse. All the animals were kept as long as possible and autopsied shortly before or after death. The mean death day of mice was evaluated from five to ten data of the life period of mice injected the sample solution. The confirmation of the infection of CJD was performed through the pathological studies and from the immunohistochemical detection of PrPCJD⁹⁾.

(b) Filtration ; The dead end filtration was carried out under the constant transmembrane pressure of 180 mmHg at 20°C.

(c) Treatment of surfactant ; A part of the original CJD infectious solution was added the surfactant "Sarkosyl" (manufactured by Tokyo Kasei Ind. Co. Ltd., *N*-Lauroylsarcosine Sodium Salt) to 1 wt% and was kept as it was at 20°C for 2 hr before filtration.

RESULTS AND DISCUSSION

Filtration Characteristics of BMM

In order to check the purity of the original CJD infectious solution, the ratio of the filtration rate J between pure water and the solution in question. When this ratio is higher, the purity higher also. The empirical equation obtained was as follows :
When $\dot{\gamma} = 0 \text{ sec}^{-1}$ (i. e., dead end filtration)

$$\text{pure water } J = 0.344 (2\bar{r}_f)^2 \quad (1)$$

$$(\text{ml}/\text{m}^2 \cdot \text{hr} \cdot \text{mmHg} \cdot \text{nm}^2)$$

CJD infectious solution

$$\begin{aligned} &\text{at } 10 \text{ l}/\text{m}^2 \text{ of filtration volume,} \\ &J = 0.042 (2\bar{r}_f)^2 (\text{ml}/\text{m}^2 \cdot \text{hr} \cdot \text{mmHg} \cdot \text{nm}^2) \\ &\text{at } 0.3 \text{ l}/\text{m}^2 \end{aligned} \quad (2)$$

$$J = 0.148 (2\bar{r}_f)^2 (\text{ml}/\text{m}^2 \cdot \text{hr} \cdot \text{mmHg} \cdot \text{nm}^2)$$

When $\dot{\gamma} = 500 \text{ sec}$ (tangential flow or parallel filtration)

Fig. 1 Relationship between mean death lag day $\bar{D}_t$ and logarithmic dilution ratio :
 ◎, life span of a mouse which is attained by dilution of the original solution to 10^6 .
 ○, mean value (i. e., $\bar{D}_t$) of lag death time
 —○—, range of lag death time of mice injected a diluted solution.

$$\begin{aligned} \text{pure water } J &= 0.348 (2\bar{r}_f)^2 \\ &(\text{ml}/\text{m}^2 \cdot \text{hr} \cdot \text{mmHg} \cdot \text{nm}^2) \end{aligned} \quad (3)$$

CJD infectious solution

$$\begin{aligned} &\text{at } 10 \text{ l}/\text{m}^2 \\ &J = 0.198 (2\bar{r}_f)^2 (\text{ml}/\text{m}^2 \cdot \text{hr} \cdot \text{mmHg} \cdot \text{nm}^2) \\ &\text{at } 0.3 \text{ l}/\text{m}^2 \end{aligned} \quad (4)$$

$$J = 0.220 (2\bar{r}_f)^2 (\text{ml}/\text{m}^2 \cdot \text{hr} \cdot \text{mmHg} \cdot \text{nm}^2)$$

Here, the value of shear rate $\dot{\gamma}$ at the inner wall of hollow fiber was calculated through the following equation ;

$$\dot{\gamma} = 2 (2Q - J) / \pi (D/2)^2 \quad (5)$$

where Q , J , and D are flow rate, filtration rate, and inner diameter of a hollow fiber, respectively. At the dead end filtration, the ratio of J between

Table 1 Death day of mice caused by CJD, alive number, and values of $\bar{D}_t$ and Φ^{**}

Sample code	Preparation	Death day (day)	Alive No.	$\bar{D}_t$ (day)	Φ
0	Original	119, 134, 135, 135, 142, 142, 164	0	0	0
F 7	Filtrate by BMM 75	134, 142, 142, 157, 157, 234	0	22.3	2.7
F 6	Filtrate by BMM 60	142, 142, 169, 185, 227	3	344.4	5.1
F 5	Filtrate by BMM 50	----	4	>861.3	>5.9
F 4	Filtrate by BMM 40	168, 182, 215, 254	4	463.7	5.3
F 3	Filtrate by BMM35	----	7	>861.3	>5.9
F 73	Filtrate of F7 by BMM35	----	8	>861.3	>5.9
F 63	Filtrate of F6 by BMM35	----	7	>861.3	>5.9
F 66	Filtrate of F6 by BMM60	224, 224, 236, 301	3	569.3	5.3
SF 4	Filtrate of Salkosyl treated 0 by BMM 40	116, 116, 116, 116, 116, 125	0	-21.2	-2.7

*mean death lag time defined by eq 6 or eq 7 in the test.

**logarithmic rejection coefficient defined as $\Phi = -\log_{10} N_f/N_0$, where N_f and N_0 are particle concentrations of filtrate and filtrand, respectively.

pure water and the solution decreases from 0.43 at 0.3 l/m² to 0.12 at 10 l/m² indicating that the original solution contains a lots of particles larger than 75 nm in size (75 nm is the maximum pore size among BMM[®]s employed). In the case of the supernatant of the cell culture containing viruses the ratio shows between 0.2 and 0.6 at 3 l/m². After filtration of the original solution using BMM with 75 nm in its mean pore size (BMM 75 in short), the ratio increased to 0.95 at the filtration volume of 8 l/m². Then, we can conclude that the purity of the original solution is not so high comparing with the cell culture supernatant and after the pre-filtration using BMM 75 the purity becomes as the same level of pure water if the content of the larger particles is representable as the standard of the purity. Even after the filtration using BMM75, the filtrate shows high titer of CJD as is shown below.

The permeabilities of human albumin, γ -globulin were about 100% for all the BMM employed. These data are consistant with those reported¹⁰⁾.

Quantitative Evaluation of Concentration of CJD Causative Agent

Figure 1 shows the dependence of the mean death

lag time $\bar{D}_t$ due to CJD infection to a mouse obtained through the inoculation of the ten fold diluting solution series. Here, we calculate the value of $\bar{D}_t$ using following equation : That is,

(1) When the ratio of the dead mice to whole mice within 1000 days after injection exceeds 84% then

$$\bar{D}_t = \frac{\sum_{i=1}^n D_{t,i}}{n} \quad (6)$$

where $D_{t,i}$ is the death lag time of i-th mouse and n is the total amounts of the dead mice due to CJD for each solution. Here, the death lag time is defined as the difference time of the death date and the mean death day of the mice injected the original CJD infectious solution (in this case, 139 days).

(2) When another case of the alive ratio of more than 16% then $\bar{D}_t$ is calculated by

$$\bar{D}_t = (\sum_{i=1}^n D_{t,i} + m \times 1000) / (n+m) \quad (7)$$

where m is the total number of alive mice. The thousand days corresponds to that of the life span of a mouse. Figure 1 indicates the following empirical equation holds between $\bar{D}_t$ and logarithmic dilution ratio (in short, LDR),

$$\log \bar{D}_t = 0.5 (\text{LDR}) \quad (8)$$

When we put $\bar{D}$, value of 1000 days into eq. 8, the value of LDR is six indication that the original CJD infectious solution containing the agent of 10^6 infectious numbers/0.02 ml ($=5 \times 10^7$ Infectious No./ml). Substitution of the observed value of $\bar{D}$, for each filtrate into eq. 8, we can evaluate LDR and this value coincides with the logarithmic rejection coefficient of a filter from the definition of Φ .

Removability of CJD Infectious Agent for BMM

Table 1 summarizes the day of death caused by CJD, the total number of alive mice without the infection, the value of $\bar{D}$, and the calculated value of Φ using $\bar{D}$. Here, the longest observing day after the injection was 1107 days. When the mean pore size of BMM decreases the Φ value increases and the filtrates of BMM 35 shows no infection. In this case, the no infection of a filtrate corresponds to the removability of $\Phi > 5.9$. Consequently, the size of CJD agent is estimated to be more than 40 nm when we reduce the size into the corresponding spherical size although the agents may aggregate to a rod-shaped particle.

Aggregation State of CJD Agents

We can estimate of the aggregation state through the double filtration method using two BMM⁸⁾. From the difference in the value of F6 and F66, the effect of the second filtration using BMM 60 on the Φ value is estimated to 0.2. This value is too small comparing with that of the first filtration (i. e., 5.1). This indicates the agents exist in a poly-dispersed state in the original solution. The component that can be removed by BMM 60 has the size of ca. 60 nm judging from the Φ values of F7, F6, F5, F4 and F66 using the empirical equation relating the mean pore size and the particle size⁸⁾. The content of the larger particles is 99.99% of the total amount of the infectious agent evaluating from the Φ values of these samples. The component to be removed other than the particle with ca. 60 nm in size has the size ranging between

35 nm and 60 nm, more likely 40 nm evaluated through the Φ value of F73.

The existence of the aggregates are confirmed through the data of SF 4. That is, after the treatment of the surfactant Sarkosyl, the filtrate through BMM 40 shows about 500 times of infectious agent particles comparing with that of the original solution before the treatment. The aggregates were separated into the small pieces and their size is less than 25 nm, the particle of whose size is permeable completely through BMM 40. The increase of 500 times in the infection ability by the surfactant treatment indicates that one aggregate separates into 500 smaller particles with reciprocal cubic root of 500 (i. e., 1/8) of the original size.

CONCLUSION

The CJD infectious solution contains poly-dispersed aggregates in ordinary condition and can be removed through BMM with less than 35 nm in size. Since the treatment of surfactant with strong power of dispersion of proteins make the aggregates into smaller infectious particles, then the combination of the filtration using BMM 35 and the appropriate inactivation is recommendable against CJD infection and/or prion infection. The size of the unit particle with the infectivity is forthcoming unsolved problem.

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- (受付 1993 年 5 月 20 日)

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