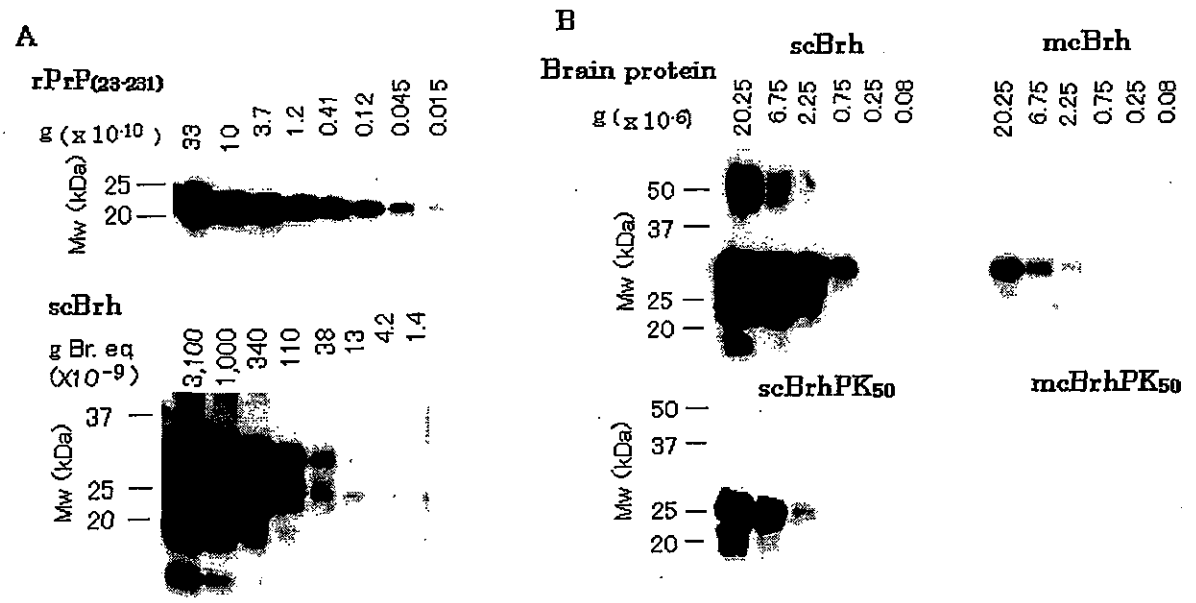

Fig. 1. Sensitivity and specificity determinations of immunoblot systems. Panel A: Sensitivity of used chemiluminescence immunoblots was determined using rPrP(25-233) (upper) or scBrh^{crude} (lower). These were diluted in the serial threefold manner as described in "Materials and Methods." Thereafter, each diluted preparation was subjected to chemiluminescence immunoblot detection. The amounts of each preparation used per lane are indicated in the figure as g ($\times 10^{-10}$) for rPrP(23-231) or g Br. eq. ($\times 10^{-9}$) for scBrh. Panel B: Specific detection of PrP in scBrh and mcBrh by chemiluminescence immunoblotting was indicated. scBrh or mcBrh (upper) or their PK-treated preparations (lower) were processed to serial threefold dilution series and subjected to chemiluminescence immunoblotting. PrP in each preparation was indicated per brain protein. Protein bands in scBrh showed the mixture of PrPres and PrPc. 3F4 and HRPGAM were used as the primary and secondary antibodies, respectively.

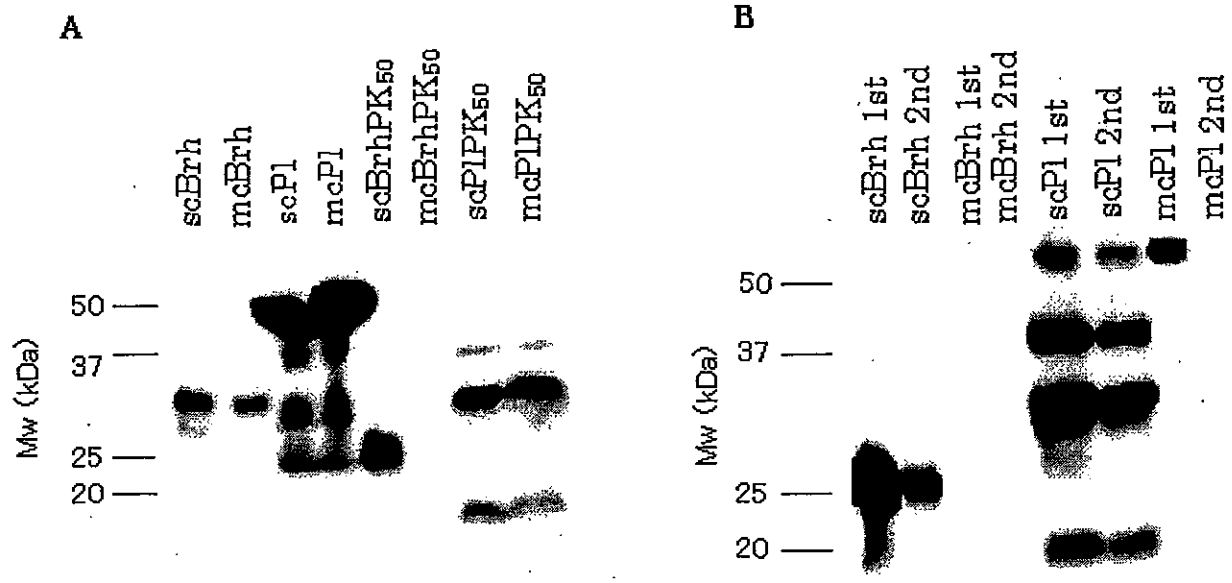

Fig. 2. Acidic SDS precipitation to discriminate scrapie infection and mock infection by their plasma. Panel A: scBrh, mcBrh, scPl and mcPl were processed and subjected to or not subjected to PK treatment. A chemiluminescence immunoblot analysis of PrP-like proteins was then performed using 3F4 primary and HRPGAM secondary antibodies. The PK treatment discriminated scBrh and mcBrh but did not discriminate between scPl and mcPl. Panel B: PK-pretreated scBrh, mcBRh, scPl and mcPl were subjected to acidic SDS precipitation condition, then analyzed by chemiluminescence immunoblotting. Acidic SDS precipitation condition was repeated twice (indicated as 1st and 2nd in the panel). This acidic SDS precipitation clearly discriminated scPl and mcPl as well as scBrh and mcBrh. The high MW protein band observed in the 1st precipitated fraction of mcPl was diminished in the 2nd precipitation. 3F4 and HRPGAM were used as the primary and secondary antibodies, respectively.

observed as in scPl. This observation was extremely different between the brain homogenate and plasma. From this observation, the presence of PK-resistant PrP molecules (PrPres-like molecules) in both sc- and mcPl was suspected (Fig. 2, panel A). These PrPres-like molecules in both plasma types have multiple inconstant Mw in experiments. Two patterns were often observed in 3F4-dependent immunoblot analysis; one was a 32 kDa major band with a 20 kDa minor band as well as 18, 25, 37 kDa faint bands (panel A; lane 7, 8), and the other was 20, 32, 40 and greater than 50 kDa dense bands as well as 27 kDa faint bands (Fig. 2, panel B; lane 5, 6).

The ability of the test to discriminate between scPl and mcPl was examined by acidic SDS precipitation (Fig. 2, panel B). PK-treated scBrh and mcBrh as well as scPl and mcPl preparations were subjected to acidic SDS precipitation condition (acidic SDS ppt) and analyzed with our immunoblotting system. In this experiment, precipitation was performed twice to ensure maximum removal of SDS soluble proteins. By this procedure, scPl and mcPl were clearly discriminated in the first precipitation and the higher Mw band that remained after the first precipitation was removed almost completely by the second precipitation. Discrimination between scPl and mcPl by acidic SDS precipitation was further confirmed by an experiment using 12 scPl and 6 mcPl samples (Fig. 3), but precipitation was only carried out once in this experiment. As shown in this figure, all 12 scPl samples showed the 3F4-reactive proteins but 4 mcPl samples did not. The mcPl of No. 1 and No. 6 showed weak 3F4-reactive bands. These observations confirm that scPl and mcPl can be successfully discriminated using the acidic SDS precipitation but that precipitation should be repeated twice. Weak bands observed in the mcPl No. 1 and No. 6 were expected to disappear by performing one more acidic SDS precipitation procedure.

Effect of Deglycosylation

It is known that three Mw species of PrP, di-, mono- and none-glycosylated molecules, exist in the brain and deglycosylation of the molecules causes the three protein species to accumulate into a single Mw. So, in order to determine whether deglycosylation affects the formation of multiple Mw protein bands in sc or mcPl, PK-treated sc and mcPl were deglycosylated or further processed using the acidic SDS precipitation procedure then compared to similarly processed scBrh. As shown in Fig. 4, 20–27 kDa proteins in scBrh and 19–50 kDa multiple Mw proteins in scPl and mcPl were detected following PK treatment (step 1). With deglycosylation of scBrh by PNGase F treatment, large amounts of 18 kDa protein appeared as was expected. Deglycosylation of scPl and mcPl resulted in 18 kDa proteins appearing but multiple higher Mw protein bands remained (step 2). After acidic SDS precipitation of these PK digested and deglycosylated materials, the multiple higher Mw protein bands in scPl disappeared, whereas a small amount of discrete 18 kDa protein bands remained in scBrh and scPl. These protein bands were not detected following similar treatment of mcPl (step 3). A long period of exposure (10 min) was necessary to obtain the protein signals described from step 3 of the experiment because the PrP-like proteins were difficult to detect after the deglycosylation step of the

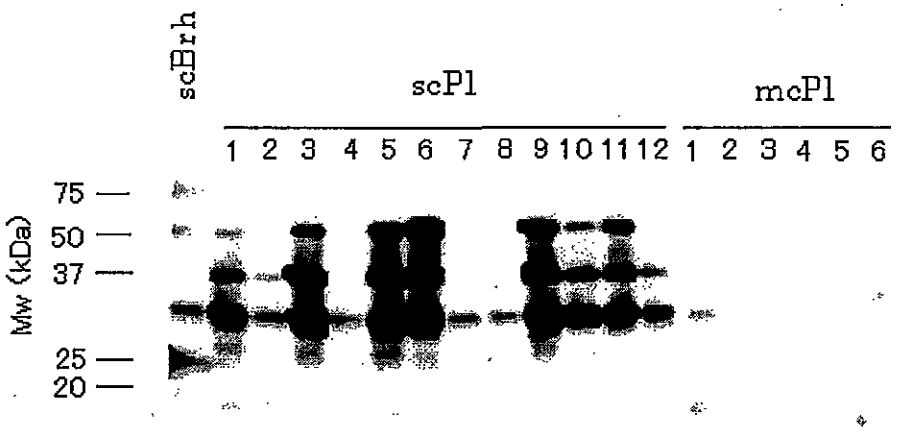

Fig. 3. Discrimination of PK-treated scPl and mcPl by acidic precipitation. Twelve preparations of scPl and 6 preparations of mcPl were pretreated with PK (50 μg/ml). scBrh was similarly treated before processing. After the pretreatment, the scPl and mcPl as well as scBrh were processed to the acidic SDS precipitation stage and analyzed by the normal immunoblot systems as described in "Materials and Methods." Anti-PrP mAb 3F4 and HRPGAM were used as the primary and secondary antibodies, respectively, for the immunoblot analysis.

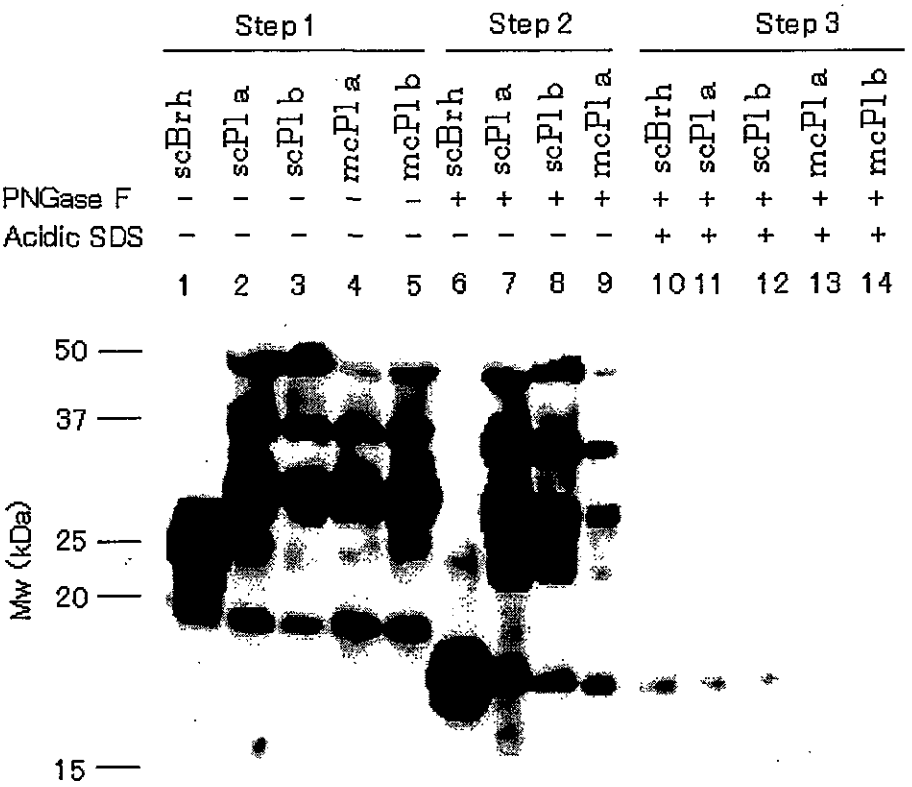

Fig. 4. Disappearance of extra Mw protein bands by digestion of carbohydrate side chains followed by acidic SDS precipitation. scBrh, two preparations of scPl and two preparations of mcPl were treated with PK (step 1, lanes 1–5). The PK-treated preparations were digested by PNGase F to remove the carbohydrate side chain on the protein molecules (step 2, lanes 6–9) then processed finally to the acidic SDS precipitation stage (step 3, lanes 10–14). Lanes were: Brain homogenate: 1, 6, 10; scPl: 2, 3, 7, 8, 11, 12; and mcPl: 4, 5, 9, 13, 14. The immunoblot pattern of each preparation during the three steps was determined. For the immunoblot analysis, 3F4 mAb and HRPGAM were used as the primary and secondary antibodies, respectively. Preparations treated with PNGase F or acidic SDS precipitation are indicated as (+) and untreated or unprocessed preparations are indicated as (−) in the figure. 3F4 and HRPGAM were used as the primary and secondary antibodies, respectively.

acidic SDS precipitation protocol. These observations suggested that carbohydrate side chains were involved in the formation of the multiple higher Mw protein bands (Fig. 4).

Appearance of Multiple Higher Mw Proteins by Mixing PK-Treated scBrh and PK-Treated or PK-Untreated mcPl

PK-treated Brh was mixed with PK-treated or PK-untreated mcPl then processed to the acidic SDS precipitation stage. These mixed preparations were compared with preparations of unmixed components in immunoblot analysis. Mixing of the PK-treated scBrh with PK-treated or PK-untreated mcPl resulted in the formation of higher Mw multiple protein bands as observed in scPl. Mixing with PK-treated mcPl seemed to show more discrete bands than mixing with PK-untreated mcPl. In PK-treated mcHaBrh, PK-treated or PK-untreated mcPl, these higher Mw protein bands were not observed. These immunoblot results suggested that the multiple Mw 3F4-reactive proteins were newly formed by the association between PrPres in scBrh and some PK-resistant plasma proteins in mcPl (Fig. 5).

Effect of Deglycosylation for the Association of PrPres in scBrh and PK-Resistant Protein in Plasma

As the deglycosylation of scPl resulted in failure to form the multiple higher Mw proteins but resulted in the appearance of a discrete 18 kDa band. As the Mw of which is similarly to the deglycosylated PrPres in scBrh, the possible involvement of saccharide chains was suspected for the formation of multiple extra Mw protein bands. To confirm this possibility, PK-pretreated scBrh and mcPl were deglycosylated by PNGase F or left untouched. After mixing the two preparations in the combination indicated in Fig. 6, acidic SDS precipitation was performed thereafter. As 3% SDS in the stored plasma or brain homogenates inhibits deglyco-

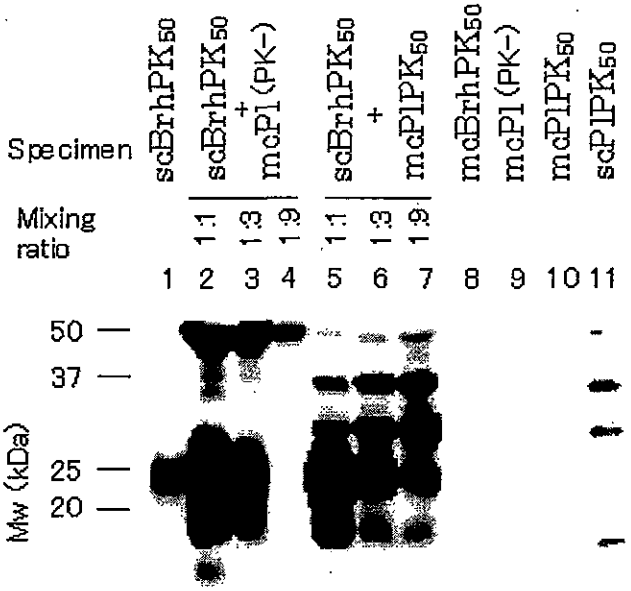

Fig. 5. Appearance of extra Mw proteins by mixing scBrh and mcPI after the acidic SDS precipitation. Proteinase K-pretreated scBrh was mixed with PK-treated or untreated mcPI and diluted to 1:3 or 1:9 in the presence of the PK-treated or untreated mcPI preparations. Then the mixed and unmixed preparations were processed to the acidic SDS precipitation stage. These processed preparations were compared by immunoblot analysis using 3F4 mAb and HRPGAM as the primary and secondary antibodies, respectively. Lanes: 1: PK treated scBrh; 2–4: PK-treated scBrh was mixed with an equal amount of PK-untreated mcPI (lane 2), diluted to 1:3 (lane 3), diluted to 1:9 (lane 4); 5–7: PK-treated scBrh was mixed with an equal amount of PK-treated mcPI (lane 5), diluted to 1:3 (lane 6), diluted to 1:9 (lane 7); 8: PK-treated mcBrh; 9: PK-untreated mcPI; 10: PK-treated mcPI; 11: PK-treated scPI. 3F4 and HRPGAM were used as the primary and secondary antibodies, respectively.

sylation reaction by PNGase F, the preparations for deglycosylation were diluted 30-fold before the reaction. After the deglycosylation, proteins in the reaction mixture of PNGase F treatment were precipitated by methanol and dissolved again to their original volumes with a primary buffer system that contained 3% SDS before mixing. Acidic SDS precipitation after the mixing of these deglycosylated preparations resulted in the appearance of an 18 kDa discrete band with a similar Mw to the deglycosylated PrPres in scBrh. Deglycosylation of brain proteins as well as of plasma proteins separately failed to form the higher Mw multiple protein bands. Mixing of PNGase F-treated scBrh and PNGase F-untreated mcPI formed a somewhat large amount of discrete 18 kDa proteins (lane 5).

Discussion

For antemortem diagnostic tests, body fluids such as blood or urine may be the most convenient specimens. The infectivity of blood in TSE-infected animals has already been determined to be 10–30 ID_{50}/ml (5). For this reason, the detection sensitivity of PrPres in blood is required to be in the order of ng/ml to test for TSE as has been mentioned elsewhere. Immunoblotting systems cannot detect such a low level of PrPres even in the blood of experimentally infected animals, so more sensitive methods to detect lower concentrations of PrP molecules need to be developed for antemortem diagnostic tests using blood or other body fluids. Various trials by several investigators have attempted to solve this extremely difficult problem (7, 19, 20, 30). In these studies, capillary electrophoresis analysis using a fluorescence-labeled synthetic PrP peptide, a combination of conformation-dependent PTA precipitation and ELISA, PCR of synthetic RNA conjugated with anti-PrP mAb and *in vitro* multiplication of abnormal PrP isoform (Protein Misfolding Cyclic Amplification; PMCA) have been suggested (3, 20, 29, 30). The PMCA method was shown to detect the presence of PrPres in scrapie-infected pre-mortem hamster blood using the buffy coat lysate (29). However, because these methods are complex and require a long time to obtain final results, their use in blood screening may be restricted. On the other hand, the common immunoblotting system used after PK treatment is excellent for detecting PrPres in the CNS or in other disease-affected tissues of infected animals. However, the usual immunoblot detection is less sensitive than the methods mentioned above. Therefore, a method that uses the common immunoblotting system would be the first choice for an antemortem test if its detection sensitivity could be enormously enhanced. It is suspected that the detection of PrPres molecules in blood is made more difficult by contamination from a large amount of protein, and so a method that will selectively concentrate the PrPres in blood to allow detection is therefore required. We tried to use the common immunoblotting systems in combination with a selective concentration method for PrPres-like protein aggregates and a highly sensitive chemiluminescence method. Using this combination, we successfully showed the presence of PrPres-like proteins in the scPI by means of reactivity to several anti-PrP mAbs, and by the similarity of Mw with the PrPres in infected hamster brains after deglycosylation. Moreover, carbohydrate may cause the PrPres-plasma protein aggregation and form the multiple Mw 3F4-reactive PrP-like proteins. PrP is a membrane protein and is known to aggregate frequently, especially after conversion to its disease-associated abnormal isoform. For this reason, detection of these aggregates is also the optimal way to develop an assay

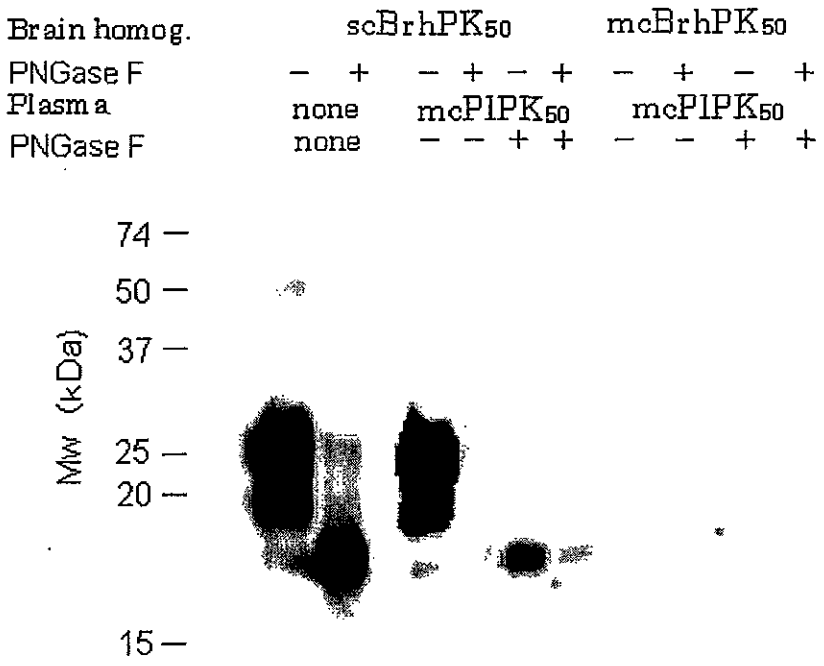

Fig. 6. Inability to form aggregate between scBrh and mcPl by digestion of carbohydrate before mixing. PK-treated scBrh, mcBrh and mcPl were further treated or not treated with PNGase F to digest the carbohydrate side chains on the proteins. These pretreated preparations were mixed with each other as indicated in the figure, and processed to the acidic SDS precipitation stage after mixing. Lanes: 1: scBrh (PNGase F−), 2: scBrh (PNGase F+); 3: scBrh (PNGase F−) mixed with mcPl (PNGase F−); 4: scBrh (PNGase F−) mixed with mcPl (PNGase F+); 5: scBrh (PNGase F+) mixed with mcPl (PNGase F−); 6: scBrh (PNGase F+) mixed with mcPl (PNGase F+); 7: mcBrh (PNGase F−) mixed with mcPl (PNGase F−); 8: mcBrh (PNGase F−) mixed with mcPl (PNGase F+); 9: mcBrh (PNGase F+) mixed with mcPl (PNGase F−); 10: mcBrh (PNGase F+) mixed with mcPl (PNGase F+), in which (PNGase F+) and (PNGase F−) mean digested or non-digested with PNGase F before mixing, respectively. 3F4 and HRPGAM were used as the primary and secondary antibodies, respectively.

method when using blood. However, previous tests for evaluating the sensitivity of detection systems using PrP molecules have frequently failed, presumably due to the tendency of the PrP molecule to form aggregates. We therefore evaluated the sensitivity of the detection system using SDS sample buffer which contained 0.1% BSA for the dilution buffer and by boiling the preparation throughout the serial dilution steps. This method allowed us to obtain a proper dispersion of the PrP aggregate in the test preparation and we successfully showed that the endpoint of the detection system was 1.5×10^{-12} g (6.05×10^{-17} mol) or more of rPrP and PrPres in 1.4×10^{-9} g brain equivalent of scHaBrh. As the scBrh has an infectivity titer of 10^{-7}–10^{-9} ID_{50}/ml, this chemiluminescence system can detect PrPres corresponding to 1 ID_{50}/ml or more, which is sufficiently greater than the value required to detect PrPres in blood (Fig. 1). We therefore decided to use this chemiluminescence system to detect PrPres in scPl. This system also allowed us to determine the detection limit of PrP protein in the brain (Fig. 1B).

Adding the acidic SDS precipitation stage to the protocol enabled successful discrimination of scPl and mcPl. The acidic SDS condition may selectively target aggregated PrP molecules, suggesting that PrP molecules in mcPl may not be aggregated. This observation is reasonable in that one of the main differences between PrPres and PrPc may be whether they exist in an aggregated form or not. Some investigators have tried to obtain PrPres in blood in an aggregated form (7). The aggregation of PrPres is thought to be a result of the more hydrophobic nature of the PrPres molecule than that of PrPc (24). However, the phenomenon observed here clearly suggests that an important factor for aggregation may be the presence of a carbohydrate side chain on both PrPres and plasma proteins rather than the hydrophobic nature of the PrPres. Carbohydrate has often been described as the outfitter for glycosylation and function (25).

The types of protein that aggregate with the PrPres-like molecules are not known. Some plasma proteins are known to associate with the PrP, but it is possible that the PrP molecules in hamster plasma may also be a candidate for these plasma proteins (11, 31, 34). PK-

resistant PrP molecules have recently been reported in uninfected human brains as well as in uninfected mouse and hamster brains and have been labeled a silent prion. PK-resistant protein in mcPl, which is able to aggregate with PrPres could be the silent prion in hamster plasma (34). Weakly observed 3F4-reactive protein bands in mcPl suggest the existence of the silent prion in plasma (Fig. 3).

In the lanes of scPl-2, -4, -7, -8, discrete bands in the Mw 32 kDa region were observed without other bands. As the band of this Mw region was weakly observed in mcPl-1 and scPl-6, it is somewhat difficult to decide the positive expression of 3F4-reactive protein for scPl-2, -4, -7 and -8. However, intensities of these signals in the scPl preparations were obviously strong compared to the signals in mcPl preparations. Thus it may be difficult to decide positive or not positive by performing acidic precipitation just one time. It is reasonable that an individual animal does not express the 3F4-reactive protein similarly in a time dependent manner and expression strength. Therefore, if blood testing is introduced, the plasma preparation should be processed twice with this acidic SDS precipitation, and the test should be conducted several times at different times.

Here we showed the successful discrimination of scrapie-infected and mock-infected hamsters by their plasma preparations using a novel combination method termed acidic SDS precipitation along with a highly sensitive chemiluminescence immunoblot system. In the immunoblots of PK-treated plasma preparations, multiple protein bands at Mw higher than the 25 kDa position were observed. These protein bands were observed in both scPl and mcPl after PK treatment. As these proteins were 3F4-reactive as well as PK-resistant, they were very likely to be PrPres molecules. However, observations showing multiple bands of higher than 25 kDa in Mw in mcPl as well as in scPl were very different from the electrophoresis pattern of scBrh. These differences between plasma and Brh have to be explained if the multiple PrPres-like proteins in plasma are aggregates of PrPres and some other plasma protein. This is similar with an observation in which the C-terminal domain of a recombinant mouse PrP peptide was aggregated spontaneously even in SDS sample buffer (24). Differences of electrophoresis patterns in Fig. 5, lanes 5–7 and Fig. 6, lane 3 or Fig. 2B, lane 5, 6 in spite of the same processing protocol may explain in which aggregation counterparts with PrPres in these plasma preparations may not be the same molecule, in preparation. After the PK treatment, an enormous amount of partial peptides was distributed in the broad Mw region if total protein was stained on WB membrane. This means that multiple partial peptides which possessing carbohydrate chains may have the potential to become the counterpart of these aggregates. We could not control the combination of the molecules. A deglycosylation experiment using both scPl and scBrh solved this question. After deglycosylation and acidic SDS precipitation, both scBrh and scPl showed a single discrete protein band at the 18 kDa Mw position. This observation strongly suggests that the carbohydrate side chain might be an important factor in the aggregation of the PrPres-like protein with some other proteins. From these observations, one of the components required to form aggregates must be the PrPres molecule but the other component need not be another PrPres molecule. That is, both self aggregation as well as aggregation of multiple hetero molecules could be resulted in the formation of the multiple Mw protein bands. Although dense bands at 25 kDa was observed in the scBrh and mcPl mixing (Fig. 5, lanes 5–7), the bands were obscure in scPl (Fig. 2, lanes 5, 6). This discrepancies between the preparations may conjectured by the differences of PrPres and plasma protein ratio. In Fig. 5, lanes 5–7, larger amount of scBrh compared to mcPl showed pattern more similar to that of scBrh, larger amount of mcPl showed more discrete band pattern after the PK treatment, in reverse. In this observation, 20 and 25 kDa protein bands were decreasing gradually along with mcPl was increasing. Therefore, it is conjectured that the 20 and 25 kDa proteins were not observed if less amounts of PrPres existed in scPl as observed in Fig. 2B, lane 5, 6.

PrPres was also found in uninfected human brains and labeled a silent prion (34). Similar molecules are likely to be present in non-infected hamsters and in mouse brains as well. If the silent prion in hamster and mouse brains is also exist in plasma, the PK-resistant 3F4-reactive proteins observed in mcPl in this experiment may be the candidate in hamster plasma. The silent prions in hamster plasma could aggregate with themselves or with other proteins to form the multiple higher Mw proteins in mcPl as well as scPl. But if the silent prion exists in mcPl, it must be discriminated through the blood tests. The acidic SDS precipitation process reported here may be useful for such trials.

So, as the PrPres molecules in hamster, 25 kDa, 20 kDa and 18 kDa proteins correspond to the di-, mono and no carbohydrate molecules, respectively. Multiple higher Mw protein bands were presumably aggregates with PrPres and other plasma proteins. The phenomenon that Mw of these aggregates were not found within a constant range indicated that counterparts of presumable PrPres might not be the specialized molecules in preparations; the silent prion may be included within these inconsistent molecules. Furthermore, we could