Selective and Efficient Immunoprecipitation of the Disease-associated Form of the Prion Protein Can Be Mediated by Nonspecific Interactions between Monoclonal Antibodies and Scrapie-associated Fibrils*

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Transmissible spongiform encephalopathies are characterized by the accumulation in brain tissues of an abnormal isoform of the prion protein named PrPsc, which is the only direct marker known for transmissible spongiform encephalopathies. Here we show that PrPsc can be specifically immunoprecipitated by using several monoclonal antibodies (mAbs) of various specificities independently of the properties of their binding site (paratope). These results strongly suggest that a significant proportion of mAbs can interact with PrPsc aggregates through nonspecific paratope-independent interactions allowing selective immunoprecipitation of PrPsc when these mAbs are immobilized on a polydisperse solid phase like microbeads.

Transmissible spongiform encephalopathies (TSE)[1] are almost always accompanied by the accumulation in the central nervous system of an abnormal form of a protein naturally produced by the host, the prion protein, PrP. This abnormal form (called PrPsc, for scrapie PrP) often accumulates in brain as amyloid plaques or deposits of which it is the major component. PrPsc is considered a hallmark for TSEs and is derived from the normal form of the prion protein (PrPc) through post-translational modifications that induce a conformational change and confer on it a partial resistance to degradation by proteases, as well as a marked insolubility in the presence of detergents. In brain homogenates or extracts, PrPsc is present as large aggregates named scrapie-associated fibrils (SAFs). PrPsc is the only direct unambiguous marker known for TSEs, and its detection is the basis of most diagnostic tests for prion diseases. Because PrPc is always present in tissues expressing PrPsc, specific detection is currently achieved after a protein-

ase K treatment, which completely degrades the cellular form of PrP and spares most of PrPsc sequence. In recent years, several papers (1–4) have described monoclonal antibodies (mAbs) that specifically immunoprecipitate PrPsc when covalently coupled to a disperse solid phase (magnetic beads or protein A-Sepharose) as seen by a Western blot analysis of the precipitate. Surprisingly, the first mAb described (15B3) was obtained after an immunization with bovine recombinant PrP, which is not supposed to mimic any of the characteristic features of PrPsc (1). The approach described by Paramithiotis *et al.* (2) is founded on the hypothesis that a linear epitope containing the YY (R or Q) motif is specifically exposed in PrPsc. Polyclonal and monoclonal antibodies produced by immunization with a synthetic peptide (CYYRRYYRYY) were shown to specifically immunoprecipitate PrPsc.

To clarify the mechanism by which mAbs can interact with SAFs as found in a TSE-infected brain, we have screened a large series of mAbs of various specificities. We have been able to show that antibodies unrelated to PrP can efficiently and selectively immunoprecipitate PrPsc *versus* PrPc from a TSE-infected brain by interactions that do not imply the paratope of the antibodies.

EXPERIMENTAL PROCEDURES

Brain Samples—Sheep brain samples were kindly provided by Olivier Andréoletti (INRA/ENVT, Toulouse, France). Scrapie-infected brain was from a VRQ/VRQ animal naturally infected with the Langlade isolate (5). Creutzfeldt-Jakob disease (CJD)-infected human brain were provided by Professor Hans A. Kretzshmar (Institut für Neuropathologie, Munchen, Germany) and human non-infected brain by Dr. Armand Perret-Liaudet (Hôpital Neurologique et Neurochirurgical, CHU Lyon, France).

In both the human and sheep brain, we verified that PrPc levels in non-infected brain were higher than in the infected brain. This clearly appears in Fig. 1 looking at the results recorded with antibodies recognizing PrPc (see Fig. 1, *A*, *Bar-224* and *B*, *Pri-308*).

Epitope Mapping—Dodecapeptides (frameshift by one residue) were synthesized on a cellulose membrane (Abimed, Langelfed, Germany) by the Spot method of multiple peptide synthesis (6) as modified by Molina *et al.* (7). This method has an advantage over an ELISA method in that all peptides are presented in the same orientation (peptides are bound to the membrane by their C-terminal residue). The reactivity of immobilized peptides was assessed by incubation with the mAbs, for 90 min at 37 °C. Alkaline phosphatase-conjugated anti-mouse IgG was used as secondary antibody (1 h at room temperature).

Coupling to Magnetic Beads and Immunoprecipitation Experiments—Antibodies (0.5 mg) were coupled to 1 ml of Dynabeads M-280 tosylated (from Dynal), and the surface of the beads was saturated with bovine serum albumin (BSA) as recommended by the manufacturer. The final concentration of beads was 10^9 beads/ml.

[1] The abbreviations used are: TSE, transmissible spongiform encephalopathies; SAF, scrapie-associated fibrils; mAb, monoclonal antibody; ELISA, enzyme-linked immunosorbent assay; BSA, bovine serum albumin; PK, proteinase K; SPIE-IA, solid-phase immobilized epitope immunoassay; CJD, Creutzfeldt-Jakob disease.

Brain tissues were homogenized (20% w/v) in 5% glucose using a ribolyzer and were first diluted 2-fold (10% homogenate) in 2× lysis buffer (10 mM Tris/HCl, pH 7.4, 10 mM EDTA, 1% deoxycholate, 0.5% Nonidet P-40) and finally were brought to 5% brain homogenate by diluting in the same lysis buffer. 5 μl of this 5% homogenate were incubated with or without 0.6 μg of proteinase K (PK) for 45 min at 37 °C. Samples were then diluted 10-fold with lysis buffer supplemented with 5 mM Pefabloc (Roche) to block PK digestion. 20 μl of beads coupled to antibodies were then added and reacted for 2 h at 20 °C with rotation. Beads were then washed three times in phosphate-buffered saline, 1% Tween 20 and heated to 100 °C in loading buffer without reducing agents. When PrPsc bound to the beads was measured using an ELISA technique (see Fig. 2), we proceeded as described above, but beads coupled to antibody or BSA were reacted with the sample overnight at 4 °C. After a washing step, PrPsc was removed from the beads by heating at 100 °C for 5 min in 25 μl of denaturing buffer C1 (from the Bio-Rad purification kit) and was diluted 10-fold in EIA buffer prior to being assayed by the two-site immunometric assay.

Two-site Immunometric Assay—The immunometric assay was performed in 96-well maxisorb immunoplates coated with the purified monoclonal anti-PrP antibody (SAF-34). 100 μl of sample were dispensed onto the wells and reacted for 2 h at room temperature. After three washes, 100 μl of acetylcholinesterase-labeled anti-PrP tracer, Bar-224 for sheep samples, and Pri-308 for human samples, were dispensed and reacted for 2 h at room temperature. After six washes, solid-phase bound acetylcholinesterase activity was determined using the colorimetric method of Ellman (8).

Preparation of SAFs Using the Bio-Rad Kit—SAFs were prepared using the Bio-Rad purification kit (Bio-Rad) designed to purify and concentrate PrPsc extracted from tissues. Briefly, 200 μl of 20% brain homogenate in sucrose 5% (w/v) were mixed with 200 μl of solution A containing proteinase K (80 μg/ml) and were incubated for 10 min at 37 °C. After the addition of 200 μl of solution B and centrifugation for 5 min at 20,000 × g, PrPsc recovered as a pellet was denatured with 25 μl of solution C1 at 100 °C for 5 min and was diluted 10-fold in EIA buffer before being assayed with the two-site immunometric assay.

Competition Experiments—Beads coupled to JEQ-254 and beads coupled to SHA-31 were prereacted for 1 h at room temperature with either the hapten JEQ2 (0-[4-(2-amino-ethyl)-2-hydroxyl-phenyl]-methyl-phenyl-phosphonate) or a synthetic peptide (Pri-4) corresponding to the 126–164 sequence of human PrP, respectively, both at a concentration of 100 μg/ml before contact with brain samples.

Western Blot—Samples eluted from beads were run on SDS-PAGE (12% resolving). Proteins were blotted on polyvinylidene difluoride membranes (Bio-Rad). Membranes were blocked with 5% nonfat dry milk. Immunoprecipitated PrP was detected with Pri-308 coupled to horseradish peroxidase for the human brain samples and with Bar-224 coupled to horseradish peroxidase for the sheep brain samples. Peroxidase activity was detected by chemiluminescence (SuperSignal West Dura, Pierce).

Denaturation Experiments—100 μl of 10% homogenate (see above) were mixed with 100 μl of 3 M guanidine HCl in phosphate-buffered saline at pH 7.4 or 3.5, and incubated for 5 h at 20 °C with rotation. Proteins were then methanol-precipitated (−20 °C for 2 h). After centrifugation at 16,000 × g for 20 min at 4 °C, the pellets were resuspended in 100 μl of lysis buffer. Before the addition of beads, the samples were brought to a 0.2% homogenate by dilution with lysis buffer.

For urea denaturation at 100 °C, 50 μl of 10% homogenate were mixed with 50 μl of 8 M urea and heated to 100 °C for 10 min. Before the addition of beads, samples were brought to a 0.2% homogenate by dilution with lysis buffer (final concentration 160 mM urea).

The Solid-phase Immobilized Epitope Immunoassay (SPIE-IA) Technique—The SPIE-IA technique was developed several years ago by some of us (9, 10). This new format was originally developed to allow the determination of low molecular weight haptens in excess reagent conditions (immunometric assays) but is also suitable for measuring proteins in a sandwich format using a single epitope (11). Here we used SPIE-IA to capture native SAF preparations with a solid-phase immobilized mAb, with the detection of captured SAFs being ensured in further steps after cross-linking of the SAF proteins, denaturation of SAF, and the detection of denatured PrP with an enzyme-labeled anti-PrP monoclonal antibody.

Briefly, monoclonal antibodies in hybridoma culture supernatants were immobilized on a solid phase (96-well microtiter plate) coated with a goat polyclonal anti-mouse immunoglobulin antibody (overnight at 4 °C). After a first washing step, infected brain homogenate (treated with or without PK) was reacted with solid-phase-bound mAbs (2 h at

room temperature). After a second washing step, solid-phase-bound SAFs were cross-linked with a monoclonal antibody using glutaraldehyde (0.1%, for 5 min at room temperature) before a third washing step. Residual glutaraldehyde reactivity was neutralized with borate trimethylamine as described previously (10) and solid-phase-bound proteins were denatured by 1 M NaOH (5 min at room temperature). After a fifth washing step, solid-phase-bound PrP was detected by reaction with an acetylcholinesterase-labeled anti-PrP mAb.

RESULTS

Screening of Monoclonal Antibodies for Their Capacity to Immunoprecipitate PrPsc—For this study, we tested 53 different mAbs of various specificities and from two immunoglobulin classes (20 IgM and 33 IgG). Twenty six of them were unable to immunoprecipitate significantly PrPc or PrPsc in preliminary experiments. We shall present here the results obtained with 27 different mAbs, which were categorized in four groups (Table I). mAbs of group A (all IgG) were raised against recombinant PrP or SAFs and all bind a PrP epitope containing the YY (R or Q) motif. Amazingly, one of these mAbs (Bar-234) actually binds an epitope containing the three YY (R or Q or D) motifs as described for mAb 15B3 (1). mAbs of group B (3 IgG and 1 IgM) were raised against PrP peptides or recombinant PrP and bind other PrP epitopes than the YY (R or Q) motif. mAbs of groups A and B were clearly shown to bind PrPc, recombinant PrP, or denatured PrPsc in previous techniques (ELISA, Western blot, immunohistochemistry). In contrast, mAbs of group C (1 IgG and 4 IgM), which were raised against the β-folded mouse recombinant PrP or native hamster SAF, were identified using a peculiar screening technique (SPIE-IA, see "Experimental Procedures") designed to detect interactions with SAFs. However, mAbs of group C failed to bind PrPc or recombinant PrP in ELISA and Western blot experiments (Table I, legend), so that if they bind SAFs it is not established that they recognize any form of PrP. Finally, mAbs of group D (5 IgG and 6 IgM) recognizing different proteins or peptides unrelated to PrP, or unrelated haptens (see Table II).

Immunoprecipitation experiments were conducted using mAbs coupled to magnetic beads and were performed on TSE-infected and non-infected brain homogenates from sheep (Fig. 1A, scrapie (*SC*)) or human (Fig. 1B, *CJD*) in the presence or absence of proteinase K treatment. Immunoprecipitates were analyzed by Western blot. The results of these immunoprecipitation experiments are presented in Fig. 1.

As expected, antibodies that were shown previously to bind PrPc or recombinant PrP (groups A and B, all of IgG isotype, excepting Bar-232) clearly immunoprecipitated PrPc in non-infected brain homogenates and gave negative results in the PK-treated non-infected brain (Fig. 1). Obviously, the results are influenced by species specificity, and, for instance, Bar-224, which does not recognize human PrP, gave negative results with human brain. The reverse situation is observed with Pri-308, which binds human PrP. Among these first series (groups A and B) six antibodies (SAF-53, SAF-61, 12F10, Bar-234, SHA-31, and 11C6) were able to immunoprecipitate PrPsc in either human or sheep infected brain homogenate treated with PK, five of which (all of them excepting 11C6) bind an epitope containing the YYR motif.

Among antibodies of group C none proved to immunoprecipitate PrPc as seen in non-infected brain homogenates (Fig. 1). However, all of them were able to immunoprecipitate PrPsc in either PK-treated or untreated infected brain homogenates (Fig. 1) in agreement with the results obtained by screening with the SPIE-IA technique (Table 1, legend).

As expected, none of the antibodies of group D proved to immunoprecipitate PrPc, but surprisingly, six of them, including four IgM (JEQ-254, His-2, EE-39, SDKP-21) and two IgG (His-22, JEQ-255), very clearly immunoprecipitated PrPsc in

TABLE I
Monoclonal antibodies directed against PrP or SAFs

Antibodies of the Pri-3 series were raised against the synthetic peptide 106–126 of human PrP (18). Antibodies of the Pri-9 series were raised against the synthetic peptide 214–230 of human PrP (18). 11C6 and 12F10 antibodies were raised against human recombinant PrP (19, 20). Antibodies of the Bar series were raised against ovine recombinant PrP. Antibodies of the SAF series were raised against proteinase K-treated and denatured scrapie-associated fibrils from Syrian hamster infected brain (263K). SAF antibodies were all identified as binding PrPc or denatured PrP (18). SHA antibodies were raised against proteinase K-treated and undenatured scrapie-associated fibrils from Syrian hamster infected brain (263K). mAbs of this series were screened according to their capacity to bind PrPc (as found in a non-infected brain homogenate) or to bind PrPsc (as found in a scrapie-infected hamster brain homogenate treated with PK) using the SPIE-IA technique (see "Experimental Procedures"). mAb SHA-31 was clearly identified as binding PrPc in Western blot and ELISA experiments, whereas SHA-9, SHA-29, and SHA-52 were shown to react only with SAFs when the SPIE-IA technique was used. Antibodies of the βS series were immunized with a mutated form of murine PrP (23–231), obtained by heterologous expression in bacteria (21). The far-ultraviolet CD analysis of the protein (pH7.0, no added denaturants) revealed an extensive β-sheet conformation, with little or no α helix present. βS antibodies were screened for their capacity to bind β-folded recombinant murine PrP as well as PrPc and PrPsc as for SHA antibodies. mAbs βS-36 and βS-43 were unable to bind any form of PrP (murine recombinant PrP, PrPc, PrP petides) when tested in ELISA or in Western blot techniques and were reactive only with SAFs as found in a scrapie-infected (263K) hamster brain using the SPIE-IA technique (see "Experimental Procedures"). Linear epitopes recognized by the different mAbs were identified as described under "Experimental Procedures." mAbs for which no linear epitope were identified were categorized as "conformational" or "unidentified" indicating that they bind a conformational epitope of PrP or another unknown antigen. mAbs were categorized in groups A, B, and C as described in the text.

Group	Monoclonal antibody	Isotype	Immunogen	Linear epitope
A	SAF-53	IgG2a	Proteinase K-treated and denatured SAF from scrapie-infected hamster brain	144-DYEDR*YYR*EN-153
A	SAF-61	IgG2a	Proteinase K-treated and denatured SAF from scrapie-infected hamster brain	144-DYEDR*YYR*EN-153
A	12F10	IgG2a	Recombinant human PrP	144-DYEDR*YYR*E-152
A	Bar-224	IgG2a	Recombinant ovine PrP	141-FGNDYEDR*YYR*-151
A	Bar-233	IgG2b	Recombinant ovine PrP	141-FGNDYEDR*YYR*E-152
A	Bar-234	IgG1	Recombinant ovine PrP	141-FGNDYEDR*YYR*-151 156-RYPNQV*YYR*PV-166 224-RESQA*YYQ*R-228
A	SHA-31	IgG1	Proteinase K-treated and undenatured SAF from scrapie-infected hamster brain	145-YEDR*YYR*E-152
B	Pri-308	IgG1	Synthetic peptide, human PrP: Pri3 (KTNMKHMAGAAAAGAVVGGLG)	111-HMAGAAAA-118
B	Pri-917	IgG1	Synthetic peptide, human PrP: Pri9 (CITQYERESQAYYQRGS)	216-TQYERE-221
B	11C6	IgG2a	Recombinant human PrP	Bind PrPc through a conformational epitope
B	Bar-232	IgM	Recombinant ovine PrP	Unidentified
C	SHA-29	IgG1	Proteinase K-treated and undenatured SAF from scrapie-infected hamster brain	Unidentified
C	βS-43	IgM	Recombinant β-folded murine PrP	Unidentified
C	βS-36	IgM	Recombinant β-folded murine PrP	Unidentified
C	SHA-52	IgM	Proteinase K-treated and undenatured SAF from scrapie-infected hamster brain	Unidentified
C	SHA-9	IgM	Proteinase K-treated and undenatured SAF from scrapie-infected hamster brain	Unidentified

TABLE II
Monoclonal antibodies of specificity unrelated to PrP or SAFs

Antibodies of the JEQ series were raised against a low molecular weight hapten (JEQ2) coupled to keyhole lympet hemocyanin (KLH). Antibodies of the His series were raised against a repeated motif of His-11 coupled to KLH. Antibodies of the Elec series were raised against the enzyme acetylcholinesterase purified from the electric organs of *Electrophorus electricus.* Elec-39 was shown to recognize a glycosidic epitope of acetylcholinesterase (22). Antibodies of the NSP series were raised against the N-terminal part of the substance P peptide coupled to KLH (11). Antibodies of the AMT-TP series were raised against 3′-amino-thymidine coupled to KLH.

Group	Monoclonal antibody	Isotype	Immunogen
D	JEQ-254	IgM	JEQ2 (0-[4-(2-amino-ethyl)-2-hydroxyl-phenyl]-methyl-phenyl-phosphonate)-KLH
D	JEQ-255	IgG1	JEQ2 (0-[4-(2-amino-ethyl)-2-hydroxyl-phenyl]-methyl-phenyl-phosphonate)-KLH
D	His-2	IgM	Polyhistidine, His[11]-KLH
D	His-22	IgG1	Polyhistidine, His[11]-KLH
D	Elec-39	IgM	Acetylcholinesterase from *Electrophorus electricus*
D	NSP-48	IgM	N-terminal part of substance P, RPKPQQGGC-KLH
D	AMT-TP 95	IgM	3′-aminothymidine triphosphate, AMT-TP-KLH
D	SDKP-21	IgM	*N*-acetyl-Ser-Asp-Lys-Pro, SDKPDC-KLH
D	TB-14A	IgG1	C-terminal fragment of the neurotoxic chain of the botulinic toxin
D	TB-15C	IgG1	C-terminal fragment of the neurotoxic chain of the botulinic toxin
D	TB-17B	IgG2a	C-terminal fragment of the neurotoxic chain of the botulinic toxin

the infected brain in the absence of or after PK treatment (Fig. 1). PrPsc aggregates in a scrapie-infected brain appeared more reactive than those in a CJD-infected brain (Fig. 1, compare *A* with *B*).

We also made the observation that a low but significant immunoprecipitation of PrP is observed in a scrapie-infected sheep brain homogenate, in the absence of PK treatment, with BSA-coated beads (Fig. 1*A*). This suggests that microbeads coated with any kind of protein can bind SAFs under appropriate conditions. This is supported by the observation that increasing the concentration of PrPsc in the sample led to an increased immunoprecipitation of PrPsc by BSA-coated beads as shown in Fig. 2 where immunoprecipitated PrP is measured using an ELISA method. The results recorded with five antibodies of unrelated specificity (TB-14A, TB-15C, TB-17C, NSP-48, and AMT-95) that behave like BSA-coated beads (Fig. 1*A*) may just reflect the presence of BSA on the beads as a saturating agent. PrPsc in CJD-infected brain homogenate also reacts with BSA-coated beads as shown in Fig. 2, but this is not visible in the Western blot experiments presented in Fig. 1*B*
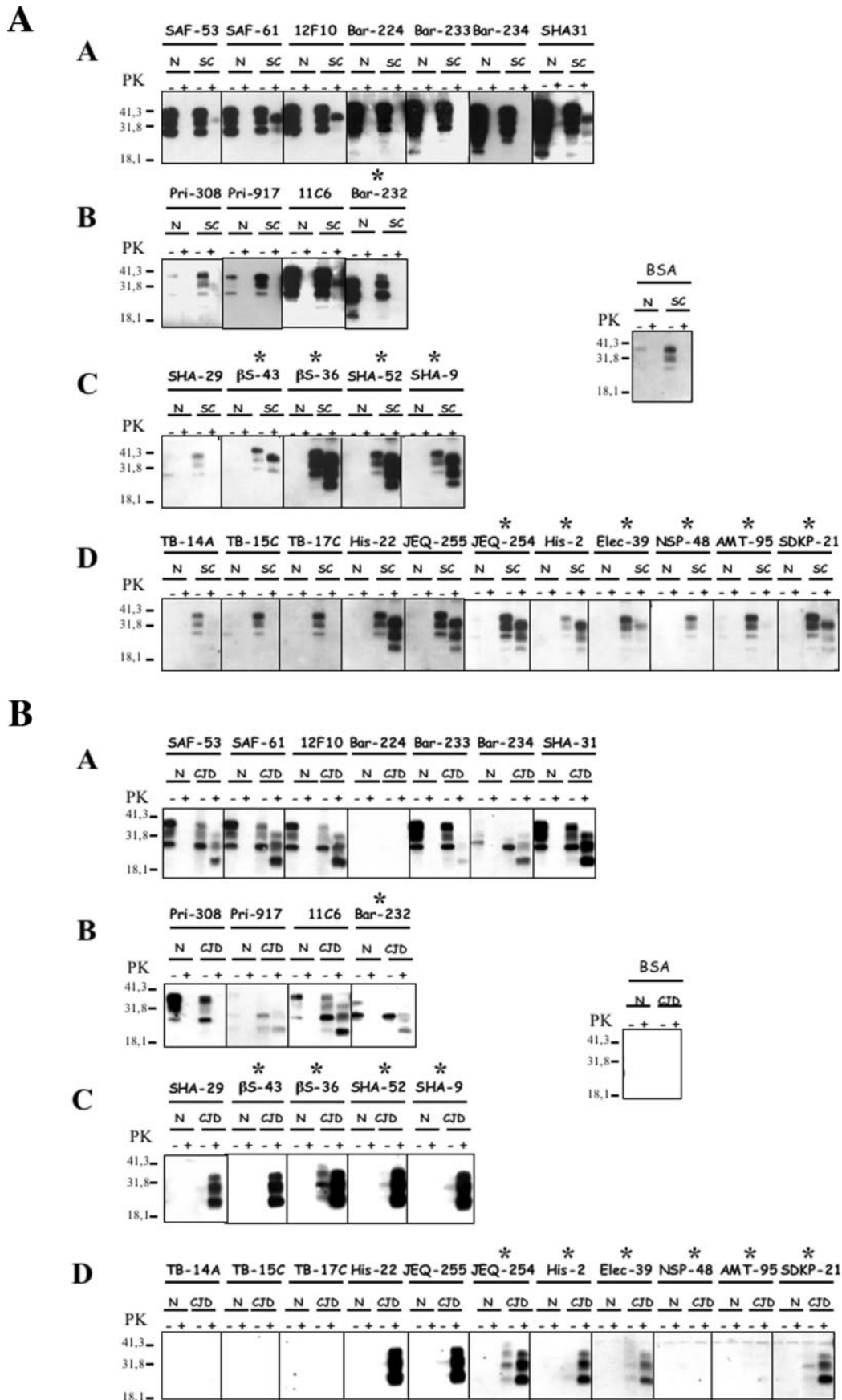
FIG. 1. **Immunoprecipitation of sheep and human PrP with different IgG and IgM monoclonal antibodies.** *A*, sheep brain, normal (*N*) and scrapie-infected (*SC*) sheep brain homogenates, treated with (+) or without (−) proteinase K (*PK*) were incubated with antibodies coupled to magnetic beads. Immunoprecipitated PrP was detected by Western blot with Bar-224 labeled with horseradish peroxidase. *B*, human brain, normal and CJD brain homogenates were immunoprecipitated as in *A* and analyzed by Western blot with Pri-308 labeled with horseradish peroxidase. The apparent molecular mass is shown in kDa.
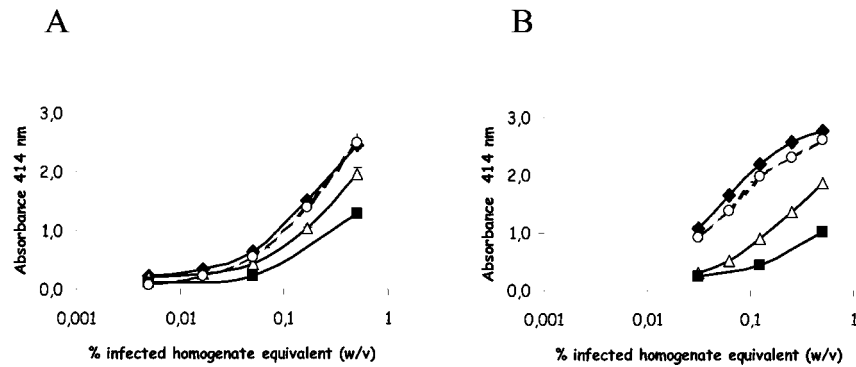
A
B

FIG. 2. **Detection of PrPsc in a TSE-infected brain.** The comparison between immunoprecipitation using beads coupled to a monoclonal antibody or BSA and a conventional method to purify SAFs (Bio-Rad purification kit) is shown. The PrPsc content of various dilutions of a scrapie brain homogenate (*A*) or CJD brain homogenate (*B*) was purified by immunoprecipitation using beads coupled to βS-36(♦), JEQ-254 (△) or BSA (■) or using the Bio-Rad purification method (○). PrPsc bound to the beads was removed from the solid phase by a denaturing treatment and measured using a two-site immunometric assay as described under "Experimental Procedures." Similarly, PrPsc contained in the SAF preparation (Bio-Rad kit) was denatured and assayed with the two-site immunometric assay.

because experimental conditions (concentration of PrPsc, reaction time) are different.

Characterization of the Interactions between PrPsc and Monoclonal Antibodies—Because these data unambiguously demonstrated that antibodies bearing specificity completely unrelated to PrP (including two antibodies recognizing an artificial haptenic molecule, JEQ2, see Table II) could react with PrPsc aggregates (SAFs), we performed competition experiments to assess whether or not this binding was mediated by the binding site of the corresponding antibody. For antibodies recognizing PrPc, a clear inhibition of PrPc immunoprecipitation was observed in the presence of an excess of the corresponding PrP peptide as shown, for instance, in Fig. 3*A* for antibody SHA-31. With this antibody a partial inhibition of PrPsc immunoprecipitation was also observed (Fig. 3*A*), indicating that the anti-PrP activity of SHA-31 participates in the immunoprecipitation process. On the other hand, no inhibition was recorded with any of the other antibodies of unrelated specificity immunoprecipitating PrPsc (His-22, JEQ-255, JEQ-254, His-2, EE-39, and SDKP-21) as illustrated, for instance, by mAb JEQ-254 in Fig. 3*B*. This shows that the immunoprecipitation process recorded with these unrelated antibodies does not involve the antibody binding site (paratope) but rather other parts of the antibody molecule. To evaluate the implication of the Fc portion of antibodies we performed the same immunoprecipitation experiments with SHA-31 and His-22 Fab′ antibody fragments. As shown in Fig. 4, SHA-31 and His-22 Fab′ fragments failed to immunoprecipitate PrPsc, whereas SHA-31 Fab′ still efficiently binds PrPc confirming that the antibody binding site is not essentially involve in the immunoprecipitation process of PrPsc.

Efficient Immunoprecipitation of PrPsc by Monoclonal Antibodies of Unrelated Specificity—Whatever the nature of the interactions between antibody-coated beads and SAFs, this may result in a very efficient immunoprecipitation of SAFs as shown in Fig. 2 where the results obtained with mAbs βS-36, JEQ-254, and BSA-coated beads are compared with those recorded using a rapid biochemical technique suitable for purifying SAFs (including a PK treatment and a centrifugation step, Bio-Rad purification kit). It is worth noting that βS-36-coated beads appear particularly efficient and immunoprecipitate the totality of PrPsc as efficiently as the biochemical method. This indicates that this antibody could be used advantageously for establishing an immunoprecipitation diagnostic test in absence of PK treatment.
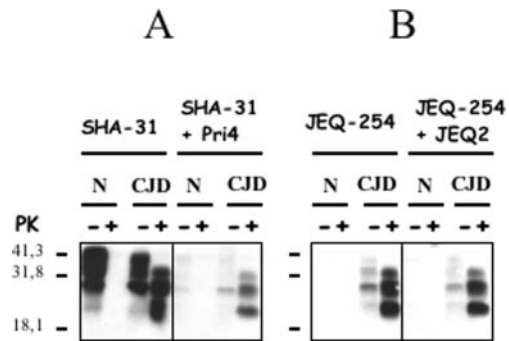

FIG. 3. **Competition experiments designed to characterize SAF-binding activity of antibodies SHA-31 and JEQ-254.** Before reaction with human brain homogenate (as described in the legend to Fig. 1), magnetic beads coupled to antibodies SHA-31 and JEQ-254 were prereacted for 1 h with or without their respective specific ligands, pri4 peptide for SHA-31 and JEQ2 for JEQ-254 (see "Experimental Procedures").
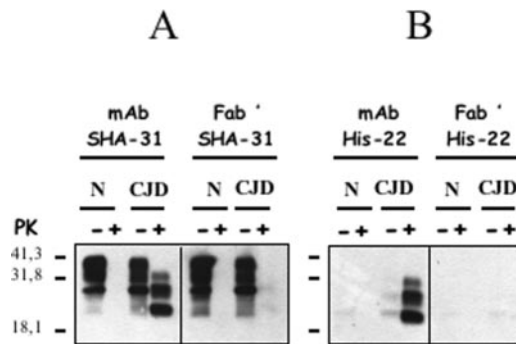

FIG. 4. **Selective immunoprecipitation of PrPsc is observed with entire antibodies but not with Fab′ fragments.** Immunoprecipitation experiments were conducted with SHA-31 and His-22 on non-infected or CJD-infected human brain homogenates, as described in the legend to Fig. 1, using either entire antibody (*A*) or Fab′ fragments (*B*) covalently bound on magnetic microbeads. *N*, normal; *CJD*, CJD-infected; +, treated with proteinase K; −, treated without proteinase K. Similar results were obtained with sheep brain homogenates from non-infected or scrapie-infected animals (results not shown).

DISCUSSION

Taken together, our results strongly suggest that SAF can be immunoprecipitated through nonspecific interactions with mAbs independently of the antibody specificity. The exact nature of these interactions has yet to be investigated but very likely involves nonspecific interactions mediated either by the constant part of the antibodies or by their sugar moiety. This is
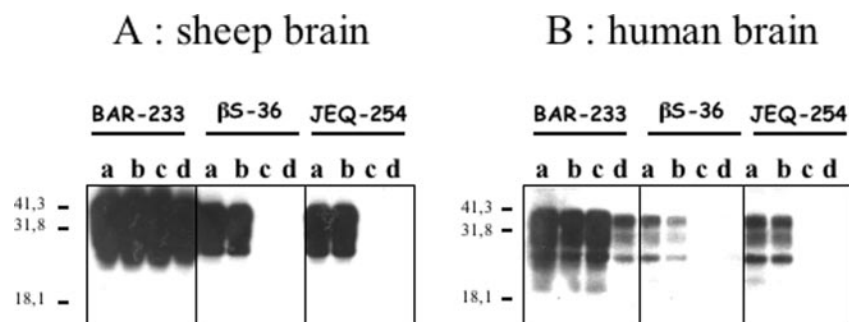
A : sheep brain   B : human brain

FIG. 5. **Immunoprecipitation of PrP from denatured or undenatured non-infected brain homogenate.** Anti-PrP antibody Bar-233 binds PrPc from treated or untreated brain homogenate, whereas βS-36 and JEQ-254 only binds PrPc from brain homogenate treated with guanidine-HCl. Before immunoprecipitation, non-infected sheep (*A*) and human (*B*) brain homogenates were denatured in different conditions with 1.5 M guanidine-HCl, pH 3.5 (*a*) or pH 7.4 (*b*), at room temperature, not treated (*c*), or treated with 4 M urea at 100 °C for 10 min (*d*).

supported by experiments showing that Fab′ fragments of some of these antibodies failed to immunoprecipitate PrPsc. This is not surprising taking into account that several proteins and polymers have already been shown to bind SAFs, including serum proteins (12), heparan sulfates (13, 14), and nucleic acids (4, 15). In addition, we show that a significant PrPsc immunoprecipitation can be obtained even with BSA-coated beads in appropriate conditions. The immunoprecipitation technique used is supposed to favor low affinity interactions, because antibodies or BSA is linked to a highly disperse solid phase (magnetic beads) providing a high density interaction surface thus favoring the binding with highly polymerized PrPsc as found in SAFs. Our data show that both IgG and IgM antibodies of unrelated specificity can ensure efficient immunoprecipitation of SAFs, but the proportion of IgM bearing this property seems to be higher. In addition, anti-PrP mAbs can participate in the immunoprecipitation of SAFs through their paratope as observed here with mAbs SHA-31 (but also with SAF-53, SAF-61, 12F10, and 11C6, results not shown) indicating that SAFs bear PrPc molecules or PrPc epitopes in agreement with previous works (16). However, our work raises serious questions concerning the actual specificity of the antibodies described previously as specifically recognizing PrPsc, because the hypothesis of nonspecific interaction between monoclonal antibodies and SAFs was not taken into account.

In the studies by Korth *et al.* (1) and Curin *et al.* (3) it is quite clear that mAbs 15B3 and V5B2 have some anti-PrP activity, because they both bind linear PrP peptides. mAb-mediated immunoprecipitation of SAFs could thus be related to low affinity anti-PrP binding enhanced by the use of microbeads, which favor the reactivity of PrPsc (largely aggregated as SAFs and presenting repeated PrP epitopes) with regard to PrPc (monomeric or oligomeric). The presence of PrPc epitopes on SAFs is supported by some observations made in our study wherein a large proportion of anti-PrP mAbs actually immunoprecipitated PrPsc, as found in PK-treated SAF preparations (Fig. 1). In this situation, the specificity of immunoprecipitation is linked to the technique used (immunoprecipitation) rather than to the intrinsic specificity of the mAbs. Alternatively, selective immunoprecipitation of PrPsc could result from nonspecific interactions as observed here with some antibodies of unrelated specificity. This question remains open and can be cleared up only by means of appropriate competitive experiments. In contrast, the rationale proposed by Paramithiotis *et al.* (2) appears highly questionable for the following reasons.

1) This paper provides very little evidence that anti-YYR antibodies actually bind any form of PrP. There are no data demonstrating that these antibodies specifically bind β-folded recombinant PrP with regards to α-folded PrP, which is the basis of the rationale described by the authors. In addition, it is

not shown that the immunizing peptide (CYYRRYYRYY) can inhibit the immunoprecipitation of SAFs. The only data supporting PrP binding are those showing that these antibodies bind "partially denatured PrP" as obtained in acidic pH and guanidine HCl-treated non-infected brain samples. To our mind, the observed binding is due rather to the aggregation of PrPc favored by this specific treatment, as shown by the same authors in a previous paper (17). This is supported by the demonstration that mAbs βS-36 and JEQ-254, which have no anti-PrP activity, were able to immunoprecipitate PrPc, as contained in a non-infected brain sample when this partial denaturing treatment was applied (Fig. 5). This confirms that immunoprecipitation is related to the aggregation of PrP and not because of an epitope specifically exposed in PrPsc or partially denatured PrPc.

2) In our study, we tested seven monoclonal antibodies directed against a linear epitope containing the YY (R or Q) motif (mAbs of group A), and most of them bind PrPc as observed in tests performed on a non-infected brain in the absence of PK treatment. It is worth noting that mAb 6H4 used as a control antibody (binding both PrPc and SAFs) by the same authors also binds an epitope containing the YYR sequence (1). This shows that the YY (R or Q) epitope is not cryptic in PrPc, which is in contradiction with the hypothesis of Paramithiotis *et al.* (2).

Our feeling is that the results reported in Paramithiotis *et al.* (2) reflect mainly nonspecific interactions between mAbs and SAFs, as those observed here with PrP-unrelated mAbs. The reasons that this is observed with all anti-YYR monoclonal antibodies described in this paper and not with BSA-coated beads and several control antibodies are not clear and possibly reflect differences under "Experimental Procedures" as well as a serendipitous selection of control antibodies.

Whatever way the antibodies were produced, it appears that a large proportion of mouse monoclonal antibodies bearing a specificity completely unrelated to SAFs or PrP allows selective immunoprecipitation of aggregated PrPsc as found in SAFs from a TSE-infected brain. In this context, the only requirement to obtain selective immunoprecipitation of PrPsc *versus* PrPc is to use antibodies not recognizing PrPc. As a consequence, such antibodies could be selected efficiently by screening large libraries of mAbs of unrelated specificity in immunoprecipitation experiments rather than resorting to more sophisticated approaches. This is very likely what we did when we screened mAbs of the βS or of the SHA series using the SPIE-IA technique (Table I, legend). Obviously antibodies bearing this property are suitable for developing a diagnostic test for TSE allowing direct detection of PrPsc as shown in Fig. 2. The actual advantages of this approach would be to allow a rapid and efficient concentration of PrPsc in the absence of PK treatment and the centrifugation step. This point is under examination in our laboratory.

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