

Polyclonal Anti-PrP Auto-antibodies Induced with Dimeric PrP Interfere Efficiently with PrP^{Sc} Propagation in Prion-infected Cells*

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Sabine Gilch^{‡§}, Franziska Wopfner^{‡§}, Ingrid Renner-Müller[¶], Elisabeth Kremmer[¶],
Christine Bauer[‡], Eckhard Wolf[¶], Gottfried Brem^{**}, Martin H. Groschup^{‡‡},
and Hermann M. Schätzl^{‡§§}

From the [‡]Gene Center Munich, Max von Pettenkofer-Institute for Virology, Ludwig-Maximilians-University of Munich, Feodor-Lynen-Strasse 25, D-81377 Munich, the [¶]Institute for Molecular Animal Breeding, Gene Center Munich, the ^{||}GSF-National Research Center for Environment and Health, Institute of Molecular Immunology, Munich, the ^{**}Ludwig-Boltzmann-Institute for Cyto-, Immuno-, and Molecular Genetic Research, Veterinärplatz 1, A-1210 Vienna, Austria, the ^{‡‡}Federal Research Center for Virus Diseases of Animals, 17498 Isle of Riems, and the ^{§§}Institute of Virology, Technical University of Munich, Biedersteinerstrasse 29, 80802 Munich, Germany

Prion diseases are neurodegenerative infectious disorders for which no prophylactic regimens are known. In order to induce antibodies/auto-antibodies directed against surface-located PrP^C, we used a covalently linked dimer of mouse prion protein expressed recombinantly in *Escherichia coli*. Employing dimeric PrP as an immunogen we were able to effectively overcome autotolerance against murine PrP in PrP wild-type mice without inducing obvious side effects. Treatment of prion-infected mouse cells with polyclonal anti-PrP antibodies generated in rabbit or auto-antibodies produced in mice significantly inhibited endogenous PrP^{Sc} synthesis. We show that polyclonal antibodies are binding to surface-located PrP^C, thereby interfering with prion biogenesis. This effect is much more pronounced in the presence of full IgG molecules, which, unlike Fab fragments, seem to induce a significant cross-linking of surface PrP. In addition, we found immune responses against different epitopes when comparing antibodies induced in rabbits and PrP wild-type mice. Only in the auto-antibody situation in mice an immune reaction against a region of PrP is found that was reported to be involved in the PrP^{Sc} conversion process. Our data point to the possibility of developing means for an active immunoprophylaxis against prion diseases.

Prion diseases, including Creutzfeldt-Jakob disease (CJD)¹ in humans, scrapie in sheep, and bovine spongiform encephalopathy (BSE) in cattle, are fatal and neurodegenerative infectious disorders. All of these diseases are characterized by the accumulation of PrP^{Sc}, the abnormally folded isoform of the cellular prion protein (PrP^C), which represents the major com-

ponent of infectious prions (1, 2). The formation of PrP^{Sc} is accompanied by profound changes in PrP^C structure and biochemical properties. PrP^C, which is rich in α -helical regions, is converted into a molecule with mainly β -sheeted structure. PrP^{Sc} becomes highly insoluble and partially resistant to proteolytic digestion (1, 3, 4). During biogenesis PrP^C transits through the secretory pathway and is modified by the attachment of two N-linked carbohydrate chains and a glycolipid anchor. The conversion of PrP^C into PrP^{Sc} is thought to occur after PrP^C has reached the plasma membrane, either at the plasma membrane or shortly after internalization in rafts (5–7). It is known that PrP^C biosynthesis is a prerequisite for PrP^{Sc} formation, and studies in transgenic animals favor a model in which PrP^C and PrP^{Sc} interact directly, possibly in combination with auxiliary factors (1, 8).

Recent work has pointed to the pivotal role of the immune system in prion infection from peripheral sites. This has been shown in various transgenic mouse models, impaired *e.g.* in B-cell maturation, FDC maturation, or in complement factors (9–12). In line with this, recent immunization studies against β A4 peptide in transgenic mouse models for Alzheimer's disease have shown dramatic and unexpected clinical and pathological improvements (13). Taken together, these data strongly suggest that a prophylactic vaccination strategy against prion infections might be a reasonable approach. In fact, anti-PrP monoclonal antibodies and recombinant Fab fragments have shown pronounced anti-prion activities in prion-infected cultured cells (14, 15). Generation of a transgenic mouse model where PrP was expressed in the presence of a defined anti-PrP antibody showed that these mice had no obvious side effects and that they were partly protected against prion infection from peripheral sites (16).

A major obstacle for an active vaccination strategy in prion diseases is the apparent autotolerance against PrP existing within a given species, which prohibits for example the production of antibodies against murine PrP epitopes within the mouse (17). This is not the case in PrP^{0/0} mice or when PrP of a different species is used as immunogen (17, 18). Characteristically, no innate or antigen-induced immune response is observed in natural prion infections (1, 2).

Here we show that it is possible to overcome the autotolerance to PrP of the own species by application of an improved immunogen, without inducing side effects that would indicate an autoimmune reaction. Recombinantly expressed dimeric mouse PrP induces highly effective polyclonal anti-PrP antibodies in rabbits and auto-antibodies in mice, which are supe-

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§§ To whom correspondence should be addressed. Tel.: 49-89-41403240; Fax: 49-89-41403243; E-mail: schatzl@lrz.tum.de.

¹ The abbreviations used are: CJD, Creutzfeldt-Jakob disease; v, variant; ELISA, enzyme-linked immunosorbent assay; PBS, phosphate-buffered saline; PK, proteinase K; BSE, bovine spongiform encephalopathy; RIPA, radioimmune precipitation assay buffer; AD, Alzheimer's disease; FA, Freund's adjuvant; mAb, monoclonal antibody; pAb, polyclonal antibody; PrP, prion protein.

rior to antibodies induced by monomeric PrP in anti-prion effects in prion-infected cultured cells. We provide evidence that full IgG molecules are much more efficient in anti-prion activity than corresponding Fab fragments, possibly by inducing a cross-linking of PrP molecules. Finally, we show that only the auto-antibodies are directed against a PrP epitope, which is usually not recognized and which is postulated to represent an interaction site involved in the conversion process of PrP (15, 19, 20).

EXPERIMENTAL PROCEDURES

Reagents—Freund's adjuvants and TiterMax were obtained from Sigma. CpG-rich oligonucleotides (1826) were synthesized from TIB Molbiol (Berlin, Germany). Proteinase K (PK) was obtained from Roche Applied Science. Immunoblotting was done using the enhanced chemiluminescence blotting technique (ECL plus) from Amersham Biosciences. mAb 4F2 has been described (21). Monoclonal anti-PrP antibody 3F4 (Signet Pathology) recognizes amino acids 109–112 of human and hamster PrP. [³⁵S]Met/Cys (Promix; 1000 Ci/mmol) was obtained from Amersham Biosciences. Protein A-Sepharose was from Amersham Biosciences. Cell culture media and solutions were obtained from Invitrogen. Disuccinimidyl suberate (DSS) was from Perbio (Bonn, Germany). PIPLC and all other chemicals were from Roche Applied Science.

Recombinant Proteins—Dimeric PrP consists of a tandem duplication of murine PrP (amino acids 23–231 = 23–231; not containing the N- and C-terminal signal peptides; amino acids 1–22 and 232–254, respectively). Murine PrP gene subunits were amplified by PCR from a 3F4-tagged full-length murine PrP using appropriate primers comprising the 7-amino acid linker sequence described before (22). Using *PvuI* sites DNA fragments were fused and cloned into the bacterial expression vector pQE30, thereby providing an N-terminal polyhistidine tag. Expression was done in *E. coli* strain BL21. Bacteria were lysed in 6 M guanidinium hydrochloride, cellular debris was removed by centrifugation at $6,000 \times g$ for 20 min, and the soluble fraction was loaded onto a Ni²⁺-column (ProBond, Invitrogen) pre-equilibrated with binding buffer (8 M urea, 20 mM sodium phosphate, 500 mM sodium chloride, pH 7.8). The column was washed several times (8 M urea, 20 mM sodium phosphate, 500 mM sodium chloride, 80 mM imidazole, pH 6.3), and the His-tagged fusion protein was eluted (8 M urea, 20 mM sodium phosphate, 500 mM sodium chloride, 500 mM imidazole, pH 6.3). Eluted fractions with highest protein concentrations were pooled, urea was removed, and PrP refolded by dialysis against ultra-filtrated water (pH 4.5). Monomeric PrP-(23–231) was similarly constructed as a 3F4-tagged murine PrP in the same vector. The genotype of both immunogens was confirmed by sequencing to be *Prnp*^{a/a}.

Immunizations—Female C57BL/6 mice (*Prnp* genotype a/a; 6 weeks old) were immunized by subcutaneous application of 50 µg of dimeric or monomeric recombinant PrP, respectively. For the classical approach at day 0 protein was applied with complete Freund's adjuvants (1:1, v/v), followed by two booster injections at days 21 and 42, using incomplete Freund's adjuvants (1:1, v/v). The immunizations with CpG (10 nmol per injection), TiterMax (1:1, v/v) and the combination of CpG and incomplete FA (10 nmol of CpG plus FA, 1:1, v/v) were only boosted once at day 21. Ten days after the last immunization blood was taken for testing of antibody reactivity. Prebleed samples were taken 7 days before starting the immunizations. Rabbits were immunized three times in the classical way using 500 µg of dimeric or monomeric recombinant PrP, respectively, and Freund's adjuvants.

Antibody Titer (ELISA)—Antibody titers were determined by an ELISA in 96-well format. Wells were coated with 1 µg of monomer or dimer, respectively, in 150 µl of carbonate buffer (0.1 M, pH 9.5) overnight at room temperature. Wells were washed with PBST (PBS, 1% Tween) and blocked with 150 µl of blocking buffer (PBST, 3% bovine serum albumin (w/v)) for 2 h at 37 °C. Wells were incubated with 100 µl of prediluted polyclonal sera for 1 h at 37 °C. After thorough washing, incubation with the appropriate mouse or rabbit conjugate (dilution 1:4,000) was performed for 1 h at 37 °C. The last washing step was followed by incubation with 100 µl of ABTS (2,2'-azido-bis[3-ethylbenzthiazidine-6-sulfonic acid]). Reaction was stopped with 1 N H₂SO₄, and optical density measured at 405 nm wavelength. Preimmune sera served as negative controls at a dilution of 1:100, and the cutoff for positive sera was calculated with 2.5 times the average extinction of preimmune sera. Titers were evaluated by end-point dilution approach (highest dilution of the sera that was still positive).

Cell Culture and Mode of Antibody Application—The mouse neuro-

blastoma cell lines N2a and ScN2a have been described (expressing wild-type murine PrP and 3F4-tagged mouse PrP; both *Prnp* genotype a/a) (23). Cells were maintained in Opti-MEM medium containing 10% fetal calf serum. Polyclonal auto-antibodies were added at a 1:50 dilution, if not otherwise stated. This dilution was determined by serial dilution studies as most appropriate for discriminating effective and non-effective antisera. Fabs were applied at a concentration of 20 µg/ml (400 nM) for overnight incubation in short term experiments. For long term treatment, medium changes were done every other day, and antibodies were added fresh with each medium change.

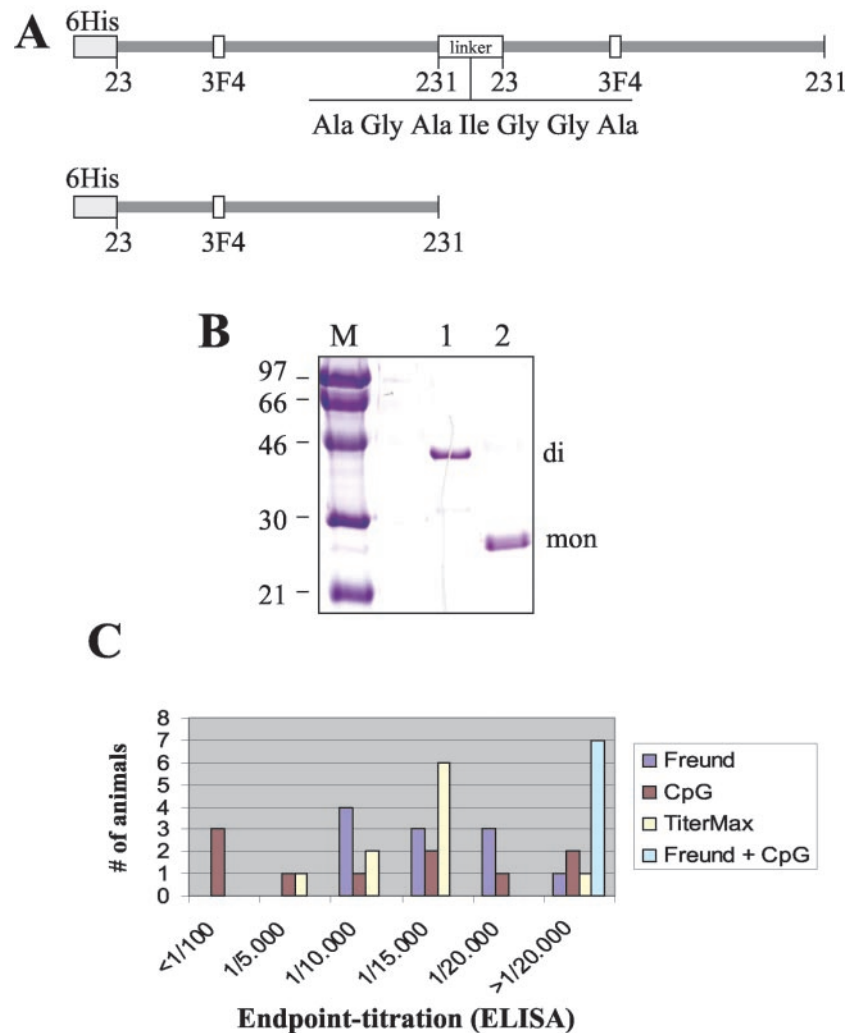
Metabolic Radiolabeling and Immunoprecipitation Assay—Confluent cells were washed twice with PBS and incubated 1 h in RPMI 1640 without methionine/cysteine containing 1% fetal calf serum. The medium was supplemented with 800 µCi of L-[³⁵S]Met/Cys for 16 h (± antisera) without a chase period. For competition assay, the antibody (1:40 in labeling medium) was preabsorbed with increasing amounts of recombinant dimeric prion protein (0–20 µg) for 1 h at 37 °C and then added to the cells. After incubation, cells were washed twice in ice-cold phosphate-buffered saline and lysed in cold lysis buffer (100 mM NaCl, 10 mM Tris-HCl, pH 7.8, 10 mM EDTA, 0.5% Triton X-100; 0.5% sodium deoxycholate); insoluble material was removed by centrifugation. For PK treatment samples were divided in half. One-half was incubated with PK (20 µg/ml) for 30 min at 37 °C, and digestion was stopped by addition of proteinase inhibitors. Then lysates with and without PK treatment were subjected to ultracentrifugation in a Beckmann TL-100 ultracentrifuge (1 h at $100,000 \times g$; TLA-45 rotor) in the presence of 1% sarcosyl. Pellets were resuspended in 100 µl of RIPA buffer (0.5% Triton X-100, 0.5% deoxycholate, in PBS) with 1% SDS, boiled for 10 min, and diluted with 900 µl of RIPA buffer (supplemented with 1% sarcosyl). The primary antibody (1:300) was incubated overnight at 4 °C. Protein A-Sepharose beads were then added for 60 min at 4 °C. The immunoadsorbed proteins were washed in RIPA buffer supplemented with 1% SDS, subjected to a deglycosylation step with PNGaseF, and analyzed on 12.5% SDS-PAGE followed by autoradiography.

Immunoblot Analysis—Confluent cell cultures were lysed in cold lysis buffer. Postnuclear lysates were subjected to PK digestion or ultracentrifugation as described above or supplemented with proteinase inhibitors (5 mM phenylmethylsulfonyl fluoride and 0.5 mM Pefabloc) and directly precipitated with ethanol. After centrifugation for 30 min at 3,500 rpm, the pellets were redissolved in TNE buffer, and gel-loading buffer was added. After boiling for 5 min an aliquot was analyzed on 12.5% SDS-PAGE followed by immunoblot as described (23).

Preparation of Fab Fragments—For preparation of Fab fragments monomer-induced and dimer-induced polyclonal rabbit antisera were desalted and total IgGs purified using the Econo Pac Serum IgG purification kit (Bio-Rad) following the manufacturer's instructions. Concentration of total IgG was determined (1.5 mg/ml). For comparison of inhibitory effects between IgG and derived Fabs, this fraction was added to the cells at a dilution of 1:25 (~400 nM). This is equivalent to the concentration of Fab fragments of 20 µg/ml (400 nM). Buffer was exchanged to 20 mM Na₃PO₄, 10 mM EDTA (pH 7.0) and volume concentrated to 0.5-ml final volume by using Vivaspin 20 columns (10,000 MWCO, Vivascience). The Fab fragments were generated by papain digestion using the ImmoPure Fab preparation kit (Perbio) following the manufacturer's instructions. The Fab fragments were collected in a final volume of 3 ml, dialyzed against ultrafiltrated water, and concentrated to a final concentration of 0.5 (dimer-induced) and 1.0 µg/µl (monomer-induced) using Vivaspin columns.

Epitope Mapping—We have designed a peptide bank consisting of 14 peptides of 20 amino acids and 1 peptide of 15 amino acids in length, respectively, with 5 amino acids overlapping to adjacent peptides, encompassing full-length mature murine PrP (23–231, shown in Fig. 5A). For coating, wells (CovaLink NH modules, Nunc) were activated with the bifunctional linker DSS in carbonate buffer (15 mM Na₂CO₃, 35 mM NaHCO₃, pH 9.6) and incubated with 100 µg of each peptide or 2 µg of recombinant dimer (control), respectively, overnight at room temperature. Wells were blocked (200 mM NaCl, 0.05% Triton X-100, 3% (w/v) bovine serum albumin, in PBS) for 30 min at room temperature, washed (200 mM NaCl, 0.05% Triton X-100, in PBS), and incubated with prediluted sera for 2 h at room temperature. After another washing step wells were incubated with the corresponding conjugate (diluted 1:4,000 with blocking buffer) for 1 h at room temperature. Upon washing and incubation with ABTS, the reaction was stopped with 1 N H₂SO₄ and documented by optical density measurement and photography with a digital camera.

FIG. 1. Recombinant prion proteins used as immunogen and auto-antibody titers. A, recombinant prion proteins were constructed encompassing mature murine PrP with an N-terminal polyhistidine tag and insertion of the 3F4 epitope. For PrP-dimer, two PrP molecules were covalently linked by a 7-residue sequence. Proteins were expressed in *E. coli*, purified from inclusion bodies under denaturing conditions, refolded, and dialyzed against ultrafiltrated water. B, purity of the dimer (lane 2) and monomer (lane 3) was determined by SDS-PAGE followed by Coomassie Blue staining. Lane 1 shows a molecular weight marker (M). Proteins were obtained at a purity of ~94% and standardized to a concentration of 1 mg/ml. C, immunization of PrP wild-type mice with recombinant PrP dimer resulted in high auto-antibody titers. Four groups of 10 mice each were immunized s.c. with FA, TiterMax, CpG, or a combination of FA and CpG (see color code depicted on the right side). Blood was taken 10 days after the last immunization. Titers of individual mice were determined by end-point dilution of pAb in ELISA using the PrP dimer as antigen (x-axis). The y-axis shows the number of antisera positive at a given dilution. Of note, three animals of the combination group had to be sacrificed before the booster immunization because of severe local abscess formation.



RESULTS

Induction of Anti-PrP Auto-antibodies in PrP Wild-type Mice—In order to overcome autotolerance against murine PrP in mice we used a covalently linked recombinant tandem duplication of murine PrP (designated as PrP dimer) as an immunogen (Fig. 1A). Linkage was done using a seven-residue linker (22), resulting in a dimer consisting of two mature PrP moieties (PrP 23–231 = 23–231). This protein and a monomeric version (PrP-(23–231)) were expressed in *E. coli* as polyhistidine fusion proteins. Upon purification on Ni²⁺-columns and refolding we typically obtained proteins with a purity of more than 94% (Fig. 1B). CD analysis showed that the secondary structure of both monomeric and dimeric PrP was in an α -helical conformation (data not shown).

We immunized PrP wild-type mice and rabbits with the recombinant monomeric and dimeric murine PrP, using initially a classical scheme with Freund's adjuvants (FA) and 50 μ g (mice) or 500 μ g (rabbits) of antigen in 3 subcutaneous (s.c.) injections. More than 90% of wild-type mice and all rabbits showed clearly detectable anti-PrP antibodies/auto-antibodies in ELISA. There was no significant difference in titers between dimeric and monomeric PrP immunogens. Of note, no obvious systemic side effects or auto-immune phenomena were observed in immunized wild-type mice. Also, when testing *e.g.* peripheral blood counts, no difference between immunized and non-immunized mice was detectable (data not shown). To optimize the antibody response we compared different types of adjuvants for the immunization with dimeric PrP. In Fig. 1C

ELISA end-point titers of a representative study in 40 mice are summarized, induced with 4 different adjuvant types (10 mice each). FA and TiterMax gave comparable results, whereas the combination of CpG-rich oligonucleotides (CpG) and incomplete FA is not applicable despite induction of very high titers because of severe local abscess formation in some animals. Studies on immunization with CpG alone will be intensified in the future because no local side effects of the immunization were observed. The anti-PrP titers were determined in ELISA by end-point dilution (x-axis). The auto-antisera generated in PrP wild-type mice showed reactivity up to dilutions of 1:20,000. The antisera generated in rabbits showed reactivity up to dilutions of >1:50,000 (data not shown). In summary, we show that the immunogen used by this group is able to effectively induce auto-antibodies against PrP in wild-type mice without inducing obvious systemic side effects.

Auto-antibodies Induced by Dimeric PrP Effectively Inhibit PrP^{Sc} Biogenesis in Infected Cells—Having found that it was possible to generate polyclonal anti-PrP auto-antibodies in PrP-expressing mice, we asked whether the induced antibodies can interfere with endogenous PrP^{Sc} synthesis in prion-infected mouse cells. This should also prove the integrity and functionality of the induced polyclonal antibodies under physiological and native conditions. We added polyclonal mouse and rabbit antibodies (pAb) to the culture medium of persistently prion-infected ScN2a cells. As readout we initially measured effects on the *de novo* synthesis of PrP^{Sc}. Cells were metabolically labeled and either rabbit (*rab*) or murine (*mo*) dimer-

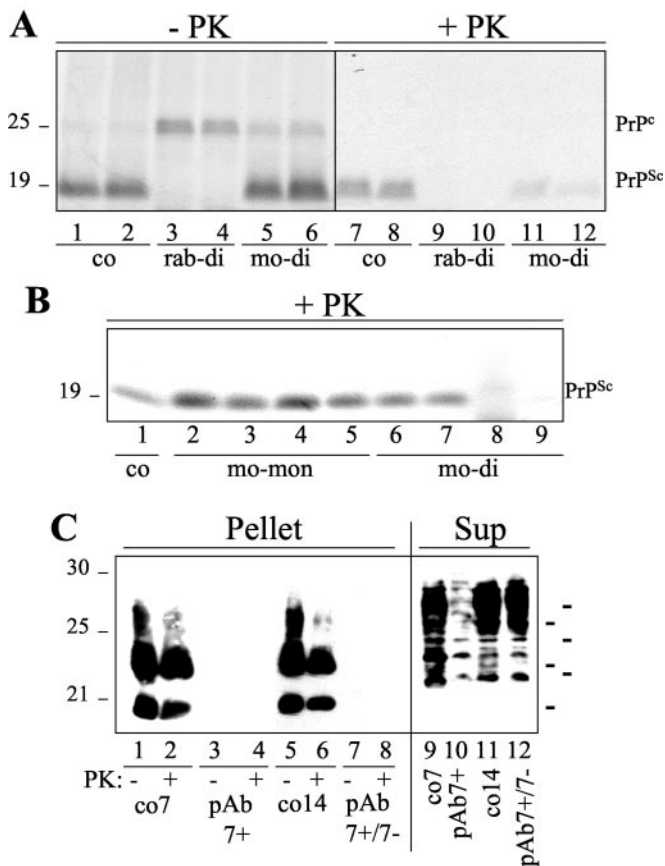


FIG. 2. Polyclonal dimer-induced anti-PrP antibodies/auto-antibodies effectively interfere with PrP^{Sc} biogenesis in prion-infected cells. A, *de novo* synthesis of PrP^{Sc} is blocked by dimer-induced rabbit anti-PrP antibodies (*rab-di*) and reduced by dimer-induced murine PrP auto-antibodies (*mo-di*). ScN2a cells were metabolically labeled with [³⁵S]Met/Cys overnight and incubated simultaneously with antibodies (at a 1:25 dilution in cell culture medium). Cells were lysed, treated $\pm$ PK, subjected to ultracentrifugation (only pellet fraction shown), and immunoprecipitated using a polyclonal anti-PrP antibody. After deglycosylation immunoprecipitates were analyzed in SDS-PAGE followed by autoradiography. The left panel shows samples without PK digestion, the right panel with PK. Control (*co*) is without antibody incubation. Cells were analyzed in duplicate. B, dimer-induced auto-antibodies from mice inhibit the *de novo* synthesis of PrP^{Sc}. Similar analysis as in A, all samples were PK-treated. Sera from individual mice (4 each) were compared (*di*, dimer-induced; *mon*, monomer-induced; pAb dilution 1:50). Control (*co*) shows cells without antibody treatment. C, long term treatment with dimer-induced auto-antibodies completely abolishes PrP^{Sc} generation. ScN2a cells were treated with pAb for 7 days. One plate was lysed (7+), and a second plate was cultivated for another 7 days without pAb (7+/7-). Lysates ($-/+$ PK) were subjected to ultracentrifugation. PrP^{Sc} signal in the pellet fraction (lanes 1–8), and the amount of PrP^c present in the supernatant (lanes 9–12) was analyzed in immunoblots and compared with mock-treated control cells (*co7* and *co14*).

induced anti-PrP antibodies were added to the medium overnight at a dilution of 1:25 (Fig. 2A). Cells were lysed ($\pm$ PK digestion), subjected to ultracentrifugation, immunoprecipitated, and analyzed in SDS-PAGE upon deglycosylation with PNGaseF (only pellet fractions shown). Antibodies induced in rabbits by dimeric PrP completely blocked PrP^{Sc} biogenesis (lanes 9 and 10), and auto-antibodies generated in mice also significantly inhibited the *de novo* synthesis (lanes 11 and 12). Interestingly, a full-length insoluble PrP population was induced by the antibody treatment that was PK-sensitive, apparently representing insoluble PrP^c ($-$ PK panel, lanes 3–6). We then compared effects exerted by auto-antibodies generated either with monomeric or dimeric PrP. Fig. 2B shows a representative analysis of 4 dimer- and 4 monomer-induced auto-

antisera generated in PrP wild-type mice (diluted 1:50). In total, sera from 26 individual mice immunized with dimeric PrP and from 19 mice immunized with monomeric PrP were analyzed for their ability to interfere with PrP^{Sc} biogenesis in cell culture. Only sera that completely blocked PrP^{Sc} *de novo* synthesis, resulting in no detectable signal for PrP^{Sc} (see lanes 8 and 9), were considered positive. We found pAb induced with dimeric PrP to be far superior in their inhibitory effect, as 12 of 26 (46.2%) were positive under these experimental conditions, in contrast to only 1 of 19 (5.3%) raised against monomeric PrP. To further verify the impact of dimer-induced auto-antibodies on the propagation of PrP^{Sc} we performed long term studies with mouse auto-antisera (Fig. 2C, left panel; dilution 1:25). ScN2a cells were treated with pAb (mouse) for 7 days, and the amount of PrP^{Sc} in the pellet fraction of the lysates ($-/+$ PK; lanes 3 and 4) was analyzed in immunoblot in comparison to mock-treated control cells (lanes 1 and 2). In auto-antibody treated cells, PrP^{Sc} was completely abolished, indicating that pre-existing PrP^{Sc} could be cleared by the cells when the *de novo* synthesis of PrP^{Sc} was blocked. Even after further cultivation of treated cells for 7 days without antibody no PrP^{Sc} was detectable (lanes 7 and 8; lanes 5 and 6, mock-treated control cells), arguing that the cells were eventually cured from PrP^{Sc} propagation by the antibody treatment. Interestingly, when we analyzed PrP^c present in the soluble fraction, the signal was decreased by the auto-antibody treatment (lane 10 versus lane 9). After another 7 days without antibody, soluble PrP^c was again expressed at the same level as in the control cells (lane 12 versus lane 11).

Taken together, our data indicate that auto-antibodies induced by dimeric PrP are able to effectively interfere with endogenous PrP^{Sc} biogenesis in prion-infected cells. After long term treatment with auto-antibodies, the amount of soluble PrP^c is significantly reduced, and the cells seem to be cured from prion infection.

Highly Superior Effect of Dimer-induced Full IgG Molecules versus Fab Fragments—Treatment of prion-infected cells with pAb/auto-antibodies resulted in a pronounced reduction of PrP^{Sc}. Assuming that this effect was mediated by a direct interaction of specific antibodies with surface PrP^c we performed competition assays (Fig. 3A). Dimer-induced pAbs were preabsorbed (1 h at 37 °C in culture medium) with increasing amounts of recombinant PrP dimer and then added to ScN2a cells simultaneously with the metabolic labeling mixture. As readout we compared the amounts of PrP^{Sc} and of insoluble full-length PrP in untreated, pAb-treated cells, and cells treated with preabsorbed pAbs. Treatment with pAb (non-preabsorbed) or with pAb after preincubation with only 1 μ g of recombinant protein (lanes 8 and 9) completely abolished *de novo* synthesis of PrP^{Sc} and induced insoluble PrP (lanes 2 and 3). Preabsorption of pAb with concentrations between 5 and 20 μ g recombinant dimer was sufficient to prevent the antibody-induced inhibition of PrP^{Sc} *de novo* synthesis, indicated by the synthesis of PrP^{Sc} in almost equal amounts compared with the untreated control cells (lanes 10–12 versus lane 7) and the absence of insoluble PrP (lanes 4–6). From this data we conclude that both the inhibition of PrP^{Sc} generation and the induction of insoluble full-length PrP were due to a specific binding of antibodies to surface PrP^c.

Given the bivalent and polyclonal character of IgG molecules and the strong binding of antibodies to PrP we speculated whether the observed induction of PrP aggregates might be the result of cross-linking of PrP^c by IgGs. To verify this hypothesis we generated Fab fragments of both monomer- and dimer-induced antisera. Due to the high amount of serum needed for the preparation this was done from rabbit sera only. The pa-

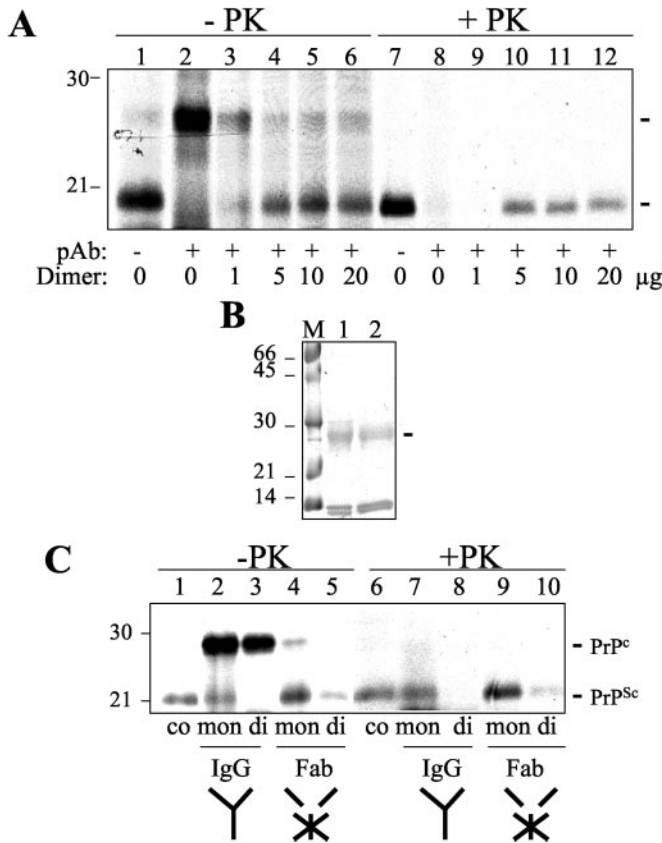


FIG. 3. Specific binding of pAb to surface PrP and highly decreased effect of Fab fragments on *de novo* synthesis of PrP^{Sc}. A, inhibition of PrP^{Sc} synthesis can be competed by preincubation of pAb *in vitro* with recombinant PrP dimer. ScN2a cells were metabolically labeled in the absence (–pAb) or presence of antibodies (+pAb) preabsorbed *in vitro* with rising amounts (0–20 μ g) of recombinant PrP dimer. Cell lysates were either left untreated (–PK) or digested with PK (+PK) and subjected to ultracentrifugation. The detergent-insoluble fraction was used for immunoprecipitation with a polyclonal anti-PrP antibody. After deglycosylation samples were analyzed in SDS-PAGE followed by autoradiography. Bars on the right indicate PrP-specific bands. B, generation of Fab fragments from polyclonal rabbit antisera. 5 μ g of Fab fragments were subjected to SDS-PAGE followed by Coomassie Blue staining. Lane 1 shows the Fab fragment derived from the monomer-induced serum, lane 2 the dimer-induced Fab. The bar on the right indicates a Fab fragment-specific band. C, reduced interference of Fab fragments with PrP^{Sc} *de novo* synthesis. ScN2a cells were metabolically labeled for 16 h and simultaneously treated with preimmune serum (co), monomer-induced (mon), or dimer-induced (di) polyclonal IgG (IgG; 1:25 (400 nM); lanes 2, 3, 7, and 8) or the corresponding Fab fragments (Fab; mon, di; 20 μ g/ml (400 nM); lanes 4, 5, 9, and 10). Lysates were divided into two samples with (+PK) or without (–PK) PK treatment. All samples were subjected to ultracentrifugation, and the insoluble fraction was used for immunoprecipitation followed by deglycosylation and SDS-PAGE. Bars on the right indicate PrP-specific bands.

pain cleavage of IgGs was confirmed by SDS-PAGE of the Fab fragments followed by Coomassie Blue staining (Fig. 3B). Only Fab molecules of about 29 kDa were visible; heavy chains (~50 kDa) were completely absent.

The efficiency of the polyclonal Fab fragments in terms of PrP cross-linking and inhibition of PrP^{Sc} synthesis compared with full IgG molecules was tested by metabolic labeling of ScN2a cells and immunoprecipitation of insoluble PrP after treatment (400 nM each, Fig. 3C). Without PK digestion, IgG molecules induced full-length insoluble PrP (lanes 2 and 3) as observed before (Figs. 2A and 3A). Interestingly, after treatment with Fab fragments no insoluble full-length PrP was detectable, which can be explained by the inability of Fabs to cross-link PrP molecules. In PK-treated lysates, only the

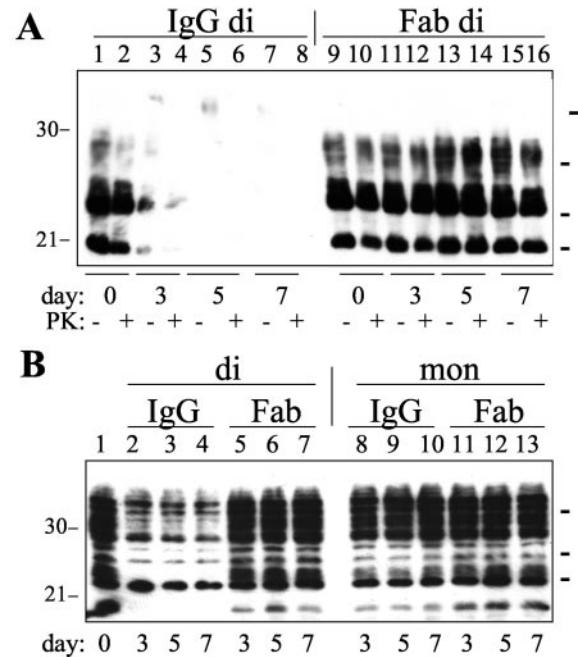


FIG. 4. Long term treatment with dimer-induced pAb eliminates PrP^{Sc} and reduces PrP^c. A, effect of long term treatment of ScN2a cells with polyclonal antisera and Fab fragments. ScN2a cells were treated with dimer-induced (di) pAb (IgG; 1:25; 400 nM) or the corresponding Fab fragments (Fab; 20 μ g/ml; 400 nM) and lysed at various time points as indicated (0, 3, 5, 7 days, respectively). Cell lysates were either treated with PK or not (–/+ PK) and subjected to ultracentrifugation. The detergent-insoluble fraction was analyzed in immunoblots using mAb 3F4. Amounts of PrP^{Sc} were compared with mock-treated control cells (0 days). B, influence on PrP^c expression after long term treatment with polyclonal antibodies and Fab fragments. ScN2a cells were treated as described in A. The detergent-soluble fraction without PK digestion was subjected to an immunoblot assay. PrP^c was detected with mAb 3F4. PrP-specific bands are indicated by the bars on the right.

dimer-induced antisera completely blocked PrP^{Sc} generation (lane 8). The corresponding Fab fragment exhibited a less pronounced effect on PrP^{Sc} synthesis (lane 10). The monomer-induced antisera, although producing almost equal amounts of insoluble PrP^c compared with dimer-induced antibodies (lane 2), had no effect on PrP^{Sc} synthesis (lane 7). The latter was also true for the corresponding Fab fragments (lane 9).

We then investigated the effect of IgG antisera and derived Fab fragments on the total PrP^{Sc} content of ScN2a cells in a long term treatment for up to 7 days. At various time points cells were lysed, treated or not treated with PK, and all lysates were subjected to ultracentrifugation. Both detergent insoluble (Fig. 4A) and soluble (Fig. 4B) PrP fractions were analyzed in immunoblot. Only upon incubation with dimer-induced full IgG molecules the cells completely eliminated PrP^{Sc} after 5 days of treatment (lanes 5–8), with the signal significantly decreased after 3 days (lanes 3 and 4). Treatment with dimer-induced Fab fragments (Fig. 4A, lanes 9–16) did not result in an inhibition of prion conversion. Of note, we used 20 μ g/ml Fab (400 nM) fragments derived from pAb, of which anti-PrP only represents a tiny fraction of total IgG (in contrast to monoclonal Fab concentrations, described in Ref. 15). As before, insoluble full-length PrP was detectable that was degraded by PK treatment (lanes 3, 5, and 7). Even in this long term study, neither monomer-induced IgGs nor the corresponding Fab fragments significantly decreased the level of PrP^{Sc} (data not shown). As already shown with the auto-antibody treatment (Fig. 2C), the amount of soluble PrP^c in cells treated with dimer-induced IgGs (Fig. 4B, lanes 3–5) was decreased compared with mock-treated control cells (lane 1). This effect was not observed upon

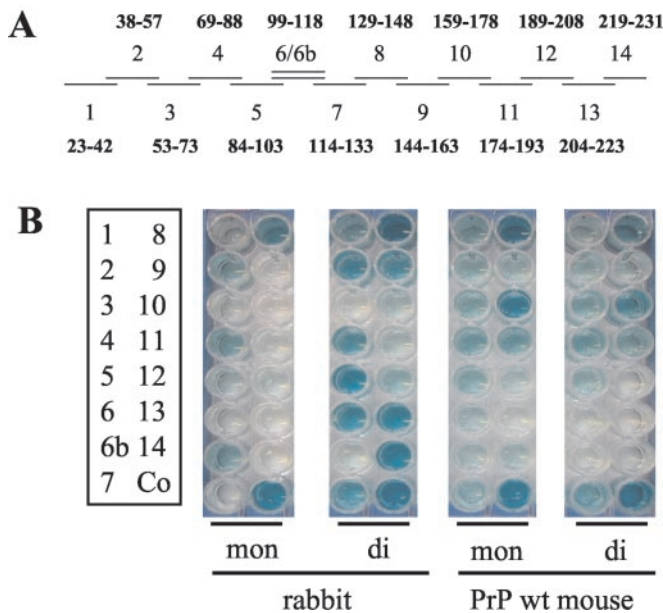


FIG. 5. Different PrP epitopes in wild-type mice and rabbits. A, peptide bank encompassing the murine PrP. Peptides consist of 20 residues, with an overlap of 5 residues. Peptide 6 encompasses the 3F4 epitope version, 6b represents the wild-type murine sequence. The positions of the peptides in the epitope mapping is indicated on the left side in B. Recombinant monomeric PrP was used as a control (Co). B, epitope mapping of representative antibodies generated by immunization with PrP monomer (mon) or dimer (di). In rabbits, dimer-induced pAb displayed high reactivity against many different N- and C-terminal epitopes, whereas monomer-induced pAb showed only reactivity against some N-terminal epitopes. Immunization with either monomeric or dimeric PrP gave a similar epitope reactivity in wild-type mice. Peptide 8 and the recombinant PrP control are recognized by all investigated pAb. Of note, peptide 10 was only reactive in wild-type mice.

treatment with monomer-induced IgGs and dimer- or monomer-induced Fabs (lanes 5–13).

In conclusion, pAbs bind specifically to surface PrP^c and can induce the formation of insoluble PrP. This cross-linking of PrP by full IgG molecules dramatically enhances the anti-prion activity compared with the effect of corresponding Fab fragments. The reduced level of soluble PrP^c in cells treated with dimer-induced IgG might add to the strong impact on the synthesis of PrP^{Sc} over time.

Auto-antibodies Are Directed Against a PrP Epitope Involved in PrP^{Sc} Conversion Process—To further characterize the polyclonal antisera we performed an epitope mapping using a peptide bank encompassing the mature full-length murine PrP (Fig. 5A). pAbs generated in rabbits and PrP wild-type mice were tested for reactivity against these peptides in an ELISA (Fig. 5B). We found that the immunization with the monomer in rabbits resulted in a very different epitope pattern compared with the pattern induced in dimer-immunized rabbits, with many more C-terminal epitopes recognized by the latter. The PrP wild-type mice responded with a similar epitope pattern regardless of the immunogen used, but with a completely different pattern compared with the rabbits. Of note, non-linear and conformational epitopes cannot be detected in our assay. Interestingly, only in the auto-antibody situation we observed a reactivity against epitope 10, which encompasses the second PrP β -sheet. This epitope is also absent in antisera of PrP^{0/0} mice that were immunized with the PrP dimer (data not shown). In summary, we demonstrate that anti-PrP auto-antibodies in mice are directed against a PrP epitope, which is apparently not recognized in rabbit and PrP^{0/0} antisera.

DISCUSSION

The rise and spread of the variant of CJD (vCJD), which is causally linked to BSE in the United Kingdom and several other countries has fueled the discussion on therapeutic and prophylactic tools against human prion disorders. Of particular interest is that vCJD, in contrast to other human prion diseases, comes with a pronounced lymphoreticular tropism, which might pose the risk of accidental transmission within human population. Therefore, prophylactic approaches are urgently needed. The classical prophylactic strategy against infectious diseases is active immunization. This is hampered in prion diseases for several reasons, one of which is the obvious autotolerance against PrP^c and PrP^{Sc}. Here we demonstrate that it is possible to induce effectively auto-antibodies directed against surface-located PrP^c and that these auto-antibodies have the potential to interfere with PrP^{Sc} biogenesis in prion-infected cells.

Auto-antibodies Against PrP^c Can Be Induced in Wild-type Mice—We provide evidence that the dimeric PrP as introduced by us might represent a prototype immunogen useful for developing active immunization strategies against prion disorders. Auto-antibodies directed against β A4 peptide were recently described as effective against pathogenic events in experimental models of Alzheimer's disease (AD) (13, 24, 25). These antibodies were apparently able to cross the blood-brain-barrier in AD animal models (13). As a note of caution, a recent clinical trial in AD patients had to be stopped because of side effects. Our experimental strategy intends to generate auto-antibodies reacting against and binding to surface-located PrP^c. Importantly, in our studies in wild-type mice we did not observe any obvious side effects. Studies in mice have shown that PrP^c can be removed without inducing side effects or neurodegeneration (26, 27). We have shown before that compromising surface PrP^c expression can be useful in prophylaxis of prion diseases (23). Similar results were obtained recently in prion-infected cells when using RNA-aptamers directed against surface PrP^c (28). A preceding surface expression of PrP^c is known to be a necessary requirement for cellular prion biogenesis (5–7), and peripheral PrP^c expression is absolutely indispensable for the transport of prions from peripheral sites of the body to the central nervous system (29). Our short term studies show that it is not a net decrease of surface-located PrP^c, needed as substrate for prion conversion, which is responsible for the inhibition of PrP^{Sc} biogenesis. On the other hand, our long term experiments with the most effective antisera have demonstrated that the steady-state level of soluble PrP^c can be significantly decreased over time.

Using various adjuvant formulations we found that it is possible to induce high anti-PrP titers (~1:20,000) without the risk of systemic side effects. Of note, the genetic PrP background of immunogens, mice and ScN2a cells employed in this study was confirmed by sequencing to be identical (*Prnp*^{0/a}). However, up to now it is not clear whether a sole antibody response will be sufficient or more importantly a strong involvement of T-cell and innate immunity will be needed for a prophylactic effect of immunization (30). Recent data have shown that co-administration of CpG alone was sufficient to prolong prion disease development after peripheral infection in a postexposure approach (31). At present it is not known whether this effect is due to activation of innate immunity, thereby clearing the prion load, or whether this is by inducing an anti-PrP antibody or T-cell response.

Why is dimeric PrP a better immunogen than monomeric PrP? Although dimeric PrP shows a very similar α -helical secondary structure when compared with monomeric PrP in CD studies, we assume that the covalent linkage forces the PrP

dimer to adopt a slightly different folding compared with authentic PrP^c or monomeric recombinant PrP, showing similarity to folding intermediates occurring during the conversion process. This might allow a better recognition of this molecule by the immune system. Evidence for the impact of the structure was obtained by using slightly degraded PrP dimers for immunization which showed, in contrast to similarly treated monomeric PrP, a drastic reduction in immunogenicity (data not shown). Of note, the difference between monomeric and dimeric PrP auto-antigen was not reflected by the antibody titers as measured with denatured PrP in ELISA. ELISA titers both for monomer- and dimer-induced murine auto-antibodies were almost equal. On the other hand, 46.2% (12/26) of dimer-induced, ELISA-positive sera from individual mice were positive in our cell culture assay compared with 5.3% (1/19) monomer-induced individual sera, which proves to be highly significant (p value = 0.003, χ^2 -test). These data clearly indicate that dimer-induced auto-antibodies have a more pronounced antiprion effect, which is not reflected by the ELISA titer. The obvious independence of ELISA titers and effects in cell culture was also evident from comparing antibodies induced in rabbits and PrP wild-type mice. Whereas much higher ELISA titers were obtained in rabbits (>1:50,000, compared with ~1:20,000 of auto-antibodies), this fact was not reflected by antiprion effects exerted in cell culture in titration studies (data not shown).

Taken together, our data demonstrate that it is possible to overcome the autotolerance against PrP^c by an active auto-immunization strategy. We show that there is no direct correlation between ELISA titer and anti-prion effect as exerted in prion-infected cell culture, but rather it depends on the structure of the immunogen and thereby displayed epitopes.

Only Full IgG Molecules Induce Cross-linking and Reduction of Soluble PrP^c—Previous studies with recombinant Fab fragments directed against PrP have shown remarkable antiprion effects in persistently prion-infected cultured cells (15). The Fab D18 reacting against PrP residues 132–156 could cure the cells. Of note, long term treatment of cells for up to 3 weeks did not result in a detectable reduction of PrP^c. In line with this are recent reports using the commercial monoclonal anti-PrP antibody 6H4 in infected cells (14) and a transgenic mouse model where PrP^c was expressed together with a specific anti-PrP antibody (16). Here again, no effects on PrP^c levels were found. Of note, concentrations of these specific monoclonal antibodies/Fabs used in both cell culture studies mentioned above for long term treatment were quite high (in the microgram range, 10–20 μ g/ml). Such concentrations are far above blood concentrations, which can be achieved by active immunization or even by passive antibody transfer in mammals.

Our studies demonstrate a striking difference in effects exerted by polyclonal full IgG molecules compared with derived Fab fragments. First, application of full IgG antibodies resulted in a pronounced induction of an insoluble full-length PrP population, which was not detectable using the homologous Fab fragments. Second, the biological effect of Fabs in cell culture on biogenesis and levels of PrP^{Sc} was highly reduced. Third, only dimer-induced pAbs decreased the levels of soluble PrP^c in long term studies. We assume that these differences result from the ability of full IgG molecules to efficiently cross-link adjacent surface-located PrP molecules. Whereas Fab fragments do not have this possibility and mAbs can only dimerize PrP using the single given epitope, pAb can use a variety of different adjacent epitopes for such a cross-linking mechanism, yielding eventually to the generation of PrP aggregates. Obviously, these aggregates are rapidly degraded by the cells. Even after treatment for 7 days with pAb we did not observe accumulation of insoluble PrP^c. Of note, also monomer-induced

rabbit antisera were able to induce a cross-linking of PrP but were not effective in reducing PrP^{Sc}. In epitope mapping, monomer-induced pAb were found to be reactive only against several N-terminal epitopes, whereas dimer-induced sera recognized N- as well as C-terminal epitopes. This indicates that cross-linking of PrP is only sufficient to decrease PrP^{Sc} when PrP^c sites implicated in prion conversion are targeted. The importance of C-terminal regions in prion conversion is supported by previous findings in cell-free conversion studies where an antibody against a C-terminal epitope (amino acids 219–232) inhibited prion conversion not by direct interference, but by sterically blocking neighboring binding sites necessary for PrP^c-PrP^{Sc} interaction (32). On the other hand, Fab fragments prepared from dimer-induced pAb recognize exactly the same epitopes as the full IgGs but are less effective in cell culture. Therefore we conclude that cross-linking significantly improves the anti-prion activity. It remains to be established whether this mechanism comes into force in an *in vivo* situation. Taken together, our data demonstrate the striking difference in molecular mechanisms used by various types of anti-PrP antibodies and underline the very special potential of polyclonal anti-PrP antibodies.

Anti-PrP Auto-antibodies Are Directed Against an Epitope Involved in PrP Interactions—A decisive factor in the efficacy of anti-PrP antibodies are the epitopes against which the reactivity is directed. In studies describing the experimental generation of anti-PrP monoclonal antibodies it was found that the PrP region encompassing residues 130–156 is a prominent epitope (21). This region of PrP has been found to be critical to prion propagation and transmission (33, 34), which was reinforced in experiments using recombinant Fab fragments in prion-infected cells (15).

To explain the molecular mechanism for the efficacy of the above mentioned Fab D18 the authors argued that the targeted epitope is exactly on the face of the PrP molecule, which is believed to be involved in PrP-PrP interaction and which is opposite to the postulated binding site for a putative cellular cofactor involved in prion conversion (15). Our epitope mapping data of anti-PrP auto-antibodies clearly indicate a different molecular mechanism in our studies. Whereas the epitope mentioned before is found in all anti-PrP preparations tested by us, we detected reactivity in auto-antibodies only against an epitope not found in heterologous immunizations, indicating a low immunogenicity in this situation. Of note, in PrP^{0/0} mice this epitope is not represented, arguing against a species-specific epitope. The epitope encompasses PrP residues 159–178 harboring the second β -sheet and one portion of the discontinuous epitope mapped previously as binding site of a putative cellular cofactor (residues Gln-168 and Gln-172, Ref. 20; numbering according to Ref. 35). This cofactor has been characterized initially in transgenic animal studies as an auxiliary cellular cofactor critical to prion propagation and to species barrier (1, 8). Interference with this putative binding activity has been discussed before as a promising anti-prion target (36). As an alternative explanation this binding site has been implicated as a mere PrP-PrP interaction site important during the seeded aggregation process (4). Experimental evidence for this hypothesis was obtained in studies using PrP peptides for interference with PrP^{Sc} formation in a cell-free conversion assay, where a peptide encompassing amino acids 166–179 was found to be a potent inhibitor by binding to PrP^c and blocking the PrP^c-PrP^{Sc} interaction (20).

Of note, the auto-antibodies induced by our auto-immunization strategy seem to target exactly this binding site. Because of the proposed interactions of this PrP region either with cellular cofactors involved in the conversion process or with

adjacent PrP molecules in prion conversion it might be usually covered and is therefore not recognized by antibodies. This might explain the complete absence of autoimmune reactions upon immunization and the lack of reactivity against epitope 10 in sera obtained from rabbits or PrP^{0/0} mice. At present, we do not know why we find this reactivity only in the autoimmune situation. One possible explanation is the selection of B-cell clones producing antibodies against PrP folding intermediates or against epitopes, which are usually covered. On the other hand, we provided experimental evidence that the induced auto-antibodies are highly effective in prion-infected cultured cells, indicating that the antibodies are able to target PrP efficiently under these conditions. The pronounced anti-prion effects, which we found for dimer-induced auto-antibodies in cell culture were in sharp contrast to that of monomer-induced antibodies, even though equal antibody titers were present. Assuming subtle differences in the folding of monomeric and dimeric PrP, this might be due to important non-linear epitopes in dimeric PrP, which we cannot measure in our epitope mapping assay. It is interesting to note that, when using dimeric PrP for generating monoclonal antibodies in PrP^{0/0} mice, we obtained various highly reactive mAbs but were not able to delineate specific linear epitopes (data not shown). This lets us argue that the dimeric PrP immunogen has a special propensity to induce discontinuous and/or conformational epitopes. One still has to evaluate whether pre-existing anti-PrP auto-antibodies will have the potential to block or reduce the invasion process of peripherally applied prions in humans and in animals. Proof-of-principle for such a potential was recently obtained by Heppner *et al.* (16). In their transgenic mouse model PrP^c was expressed in the presence of an anti-PrP antibody without inducing obvious side or auto-immunity effects (16). Noteworthy, challenge of these mice by intraperitoneal prion infection resulted in significant reduction of peripheral prion propagation. In line with this, recent immunization studies in PrP wild-type mice followed by prion challenge showed a similar although much less pronounced outcome (37).

We have described here the potential to induce anti-PrP auto-antibodies *in vivo* and their pronounced anti-prion effects in prion-infected cultured cells. Our studies provide a solid and interesting experimental basis for further active immunization and vaccination studies. Given the scenario of increasing vCJD cases in future years and the possibility that vCJD might have already been transmitted within the human population, the development of prophylactic and therapeutic approaches against prion diseases is mandatory.

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Polyclonal Anti-PrP Auto-antibodies Induced with Dimeric PrP Interfere Efficiently with PrP^{Sc} Propagation in Prion-infected Cells

Sabine Gilch, Franziska Wopfner, Ingrid Renner-Müller, Elisabeth Kremmer, Christine Bauer, Eckhard Wolf, Gottfried Brem, Martin H. Groschup and Hermann M. Schätzl

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