Screening of 145 Anti-PrP Monoclonal Antibodies for Their Capacity to Inhibit PrPSc Replication in Infected Cells*

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Prion diseases are transmissible neurodegenerative disorders affecting humans and animals for which no therapeutic or prophylactic regimens exist. During the last three years several studies have shown that anti-PrP monoclonal antibodies (mAbs) can antagonize prion propagation in vitro and in vivo, but the mechanisms of inhibition are not known so far. To identify the most powerful mAbs and characterize more precisely the therapeutic effect of anti-PrP antibodies, we have screened 145 different mAbs produced in our laboratory for their capacity to cure cells constitutively expressing PrPSc. Our results confirm for a very large series of antibodies that mAbs recognizing cell-surface native PrPc can efficiently clean and definitively cure infected cells. Antibodies having a cleaning effect are directed against linear epitopes located in at least four different regions of PrP, suggesting an epitope-independent inhibition mechanism. The consequence of antibody binding is the sequestration of PrPc at the cell surface, an increase of PrPc levels recovered in cell culture medium, and an internalization of antibodies. Taken together these data suggest that the cleaning process is more likely due to a global effect on the PrP trafficking and/or transconformation process. Two antibodies, Sha31 and BAR236, show an IC_{50} of 0.6 nM, thus appearing 10-fold more efficient than previous antibodies described in the literature. Finally, five co-treatments were also tested, and only one of them, described previously (SAF34 + SAF61), lowered PrPSc levels in the cells synergistically.

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‡ The abbreviations used are: TSE, transmissible spongiform encephalopathy; BSE, bovine spongiform encephalopathy; CJD, Creutzfeldt-Jakob Disease; vCJD, variant of CJD; PBS, phosphate-buffered saline; mAb, monoclonal antibody; SAF, scrapie-associated fibril; IL, interleukin; PK, proteinase K; ELISA, enzyme-linked immunosorbent assay; AchE, acetylcholinesterase.
cytotoxins and porphyrrins gave the most interesting results in vivo by increasing survival time by up to 50–300% in mice (19), and only quinacrine and flupirtine are under clinical evaluation in humans (21, 22). A single patient in the UK has also received pentosan polysulfate for compassionate purposes, and additional patients are being treated with this drug to test its activity (reported by the media).

Given the few possibilities for therapeutic and/or prophylactic intervention to date, new therapeutic strategies are a major focus of research to identify new compounds that could interfere with in vivo prion propagation (16, 23–25). A recent study has suggested that soluble IgG-like dimeric PrP consisting of two moieties fused to the Fc γ tail of human IgG1 (called PrP-Fc) may represent a new class of anti-prion compounds (26).

Another promising therapy lies in the potential of active or passive immunization using anti-PrP antibodies. Although active immunization strategies are confronted with a problem of self-tolerance (27), polyclonal anti-PrP auto-antibodies can be induced with dimeric PrP in wild-type mice and interfere efficiently with PrPSc propagation in prion-infected cells (28). In addition, vaccination with recombinant mouse prion protein delays the onset of prion disease in mice (29). Another vaccination approach by passive immunization is also promising. Indeed, anti-PrP antibodies were not only shown to inhibit formation of protease-resistant PrP in a cell-free system (30) but were also shown to prevent infection of susceptible N2a cells (31) and inhibit prion replication in infected cells (32–34).

In addition, transgenic expression of the anti-PrP monoclonal antibody 6H4 in mice expressing PrP blocks prion pathogenesis upon intraperitoneal inoculation (35). The mechanism by which anti-PrP antibodies interfere with PrPSc replication is not clear, but the main hypothesis presented so far involves either a perturbation of PrP cellular trafficking/PrPc degradation (33) or a disruption of the interaction between PrPc and PrPSc (32) by the antibodies. In this second hypothesis, the 132–140 portion of PrPc is thought to be important for PrPSc binding and is considered as a privileged target for development of anti-prion drugs.

Therapy for prion diseases needs delivery of anti-prion compounds either directly into the central nervous system as for all neurodegenerative disorders or in peripheral sites after prion exposure and before the neuroinvasion by the infectious agent. Concerning the first delivery case, a recent study by Solferroni (36) showing that cross-linking PrP in vivo using anti-PrP antibodies triggers rapid and extensive apoptosis in hippocampal and cerebellar neurons has prompted extreme caution over the use of PrPc-specific inhibitors directly within the central nervous system. Passive immunization by anti-PrP antibodies is, thus, thought to be more likely applicable to prion diseases where infection occurs from peripheral sites of the body (for example, oral and intraperitoneal infection) and where propagation and transport of prions outside the central nervous system have to take place before neuroinvasion. In this regard the recent in vivo study by White et al. (37), in a murine scrapie model using passive immunization, is by far the most significant one in the field of TSE therapy; continued antibody treatment started 7 or 30 days post-intraperitoneal inoculation delayed onset of scrapie for more than 300 days compared with equivalent untreated animals.

To identify those monoclonal antibodies that are the most suited for therapeutic application and to characterize more precisely the therapeutic effect of anti-PrP antibodies, we have screened 145 different monoclonal anti-PrP antibodies produced in our laboratory for their capacity to inhibit PrPSc replication in two cell culture models constitutively expressing murine or ovine PrPSc (N2a58/22L and Rov9 cells). We identified more than 30 antibodies appearing to be effective in curing the infected cells. Some of our antibodies show an IC50 10-fold lower than those previously given in the literature. Our results confirm that a very potent inhibition is obtained with monoclonal antibodies directed against PrPc present at the surface of infected cells, indicating that targeting PrPc is beneficial for prion therapy. The main consequences of antibody binding is a sequestration of PrPc at the cell surface, an increase of PrPc levels recovered in culture medium, and an internalization of antibodies. In contradiction with previous results, we show that the cleaning mechanism seems to be epitope-independent and more likely reflects a global effect on the PrP trafficking and/or transconformation process.

MATERIALS AND METHODS

Reagents and Antibodies—Dubelcco’s modified Eagle’s medium with L-glutamine, modified Eagle’s medium with L-glutamine, phosphate-buffered saline (PBS), fetal calf serum, trypsin, and Genetin were from Invitrogen. The Bio-Rad purification kit (TeSeE® containing proteinase K, PK; buffer A, and buffer B) was used for purification of PrPSc. Doxycycline and all other chemicals were from Sigma.

Screening Procedure—Screening of mAbs for TSE Therapeutic Effect

Two hundred microliters of precipitating buffer B were then added to the infected cells. Some of our antibodies show an IC50 10-fold lower than those previously given in the literature. Our results confirm that a very potent inhibition is obtained with monoclonal antibodies directed against PrPc present at the surface of infected cells, indicating that targeting PrPc is beneficial for prion therapy. The main consequences of antibody binding is a sequestration of PrPc at the cell surface, an increase of PrPc levels recovered in culture medium, and an internalization of antibodies. In contradiction with previous results, we show that the cleaning mechanism seems to be epitope-independent and more likely reflects a global effect on the PrP trafficking and/or transconformation process.
Screening of mAbs for TSE Therapeutic Effect

PrPSc was achieved by a 10-min centrifugation at 20,000 \( \times g \) at 4 °C. The supernatant was removed, and the PrPSc was denatured with 25 \( \mu l \) of 4 M urea for 10 min at 100 °C. After incubation, each sample was vortexed, and 400 \( \mu l \) of EIA buffer (0.1 M phosphate buffer, pH 7.4, containing 0.1% bovine serum albumin, 0.15 M NaCl, and 0.01% sodium azide) were added per tube.

In addition to PrPSc determination, cell-associated PrPc and total cell-associated PrP were measured. For cell-associated PrPc determination, lysed cells were diluted in EIA buffer until 100 \( \mu l \) contained 2 \( \mu g \) of total protein. For total cell-associated PrP determination, the same dilution was performed on lysed cells previously denatured (v/v) in 8 M urea for 10 min at 100 °C. Detections of PrPSc, PrPc, and total denatured PrP were then achieved by means of an appropriate immun assay described in the next paragraph.

Two-site Immunometric Assay—96-well microtiter plates (Immuno-plate, Maxisorp, Nunc) were coated with the captured IgG2a purified monoclonal antibody 11C6 at a 10 \( \mu l \)/well concentration. The plates were then saturated with EIA buffer and stored at 4 °C until use. One hundred microliters of the different samples (containing 10 \( \mu g \) of total protein for PrPSc determination, 2 \( \mu g \) of total protein for cell-associated PrPc or total PrP determination, and 40 \( \mu g \) of non-denatured or urea-denatured total protein for cell culture medium PrPc determination) were dispensed into the wells and reacted for 2 h at room temperature. Washing plates was repeated overnight at 4 °C with 100 \( \mu l \) of a 4 units/ml tracer antibody corresponding to either SAF38 covalently coupled to AChE (EC 3.1.1.7, as described previously by Grassi et al. (45)) for N2a cell PrP determination or BAR224 AChE for Rov9 cell PrP determination. After 6 washes, solid phase-bound AChE activity was assessed by the colorimetric method of Ellman (46) performed as described previously, and absorbances at 414 nm were measured with an automatic reader (Labsystem).

Concentration-dependent Inhibition and Co-treatment Inhibition—N2a22L cells plated in 6-well plates (2 \( \times 10^5 \) cells/well) were incubated with one (concentration-dependent inhibition) or two (co-treatment) purified mAbs at various concentrations ranging from 0.001 to 50 \( \mu g/ml \) over 3 days. At the end of this period cells were collected, and PrPSc, PrPc, and denatured PrP cellular contents were determined as described. Culture media with washes plates were collected overnight at 4 °C with 100 \( \mu l \) of a 4 units/ml tracer antibody corresponding to either SAF38 covalently coupled to AChE (EC 3.1.1.7, as described previously by Grassi et al. (45)) for N2a cell PrP determination or BAR224 AChE for Rov9 cell PrP determination. After 6 washes, solid phase-bound AChE activity was assessed by the colorimetric method of Ellman (46) performed as described previously, and absorbances at 414 nm were measured with an automatic reader (Labsystem).

Time-courses Inhibition—5 \( \times 10^5 \) N2a cells plated in 25-cm² flasks were incubated for 11 days in the presence of a 10 \( \mu g/ml \) concentration of purified mAbs at different monoclonal anti-PrP mAbs. Antibodies were then removed from the culture, and cells were passaged for an additional period of 17 days without antibodies. Every 3 or 4 days confluent cells were serially split into a flask (used for the prolongation of the treatment with the antibodies). A 6-well plate was used for sandwich immunassy analysis. Antibodies were added to the culture medium after splitting of N2a, PrPSc, PrPc, and denatured PrP cellular content and medium PrPc content were measured as described above.

Cytometry Measurements—For the screening procedure, infected or non-infected confluent cells in 75-cm² flasks were washed with PBS and collected after incubation at 37 °C for 5 min with 5 ml of cell dissociation buffer (without trypsin, Sigma). Samples of 50 \( \mu l \) containing 5 \( \times 10^5 \) N2a or Rov9 cells were incubated with the primary antibody (5 or 10 \( \mu l \) at 50 \( \mu g/ml \), quantities to be in excess for cellular prion staining) for 20 min at room temperature in the dark. After washes, cells were incubated with the secondary antibody, phycoerythrin-conjugated goat anti-mouse IgG or IgM immunoglobulin (Beckman Coulter, 100-fold dilution), for 20 min at room temperature in the dark. Positive controls included cells that had not been incubated with primary and secondary antibodies (autofluorescence), cells incubated with phycoerythrin-conjugated secondary antibody alone, and cells incubated with an isotypic control primary antibody and phycoerythrin-conjugated secondary antibody. Isotypic control antibodies were irrelevant purified mAbs: oIL1–101 for IgG1 (anti-o-interleukin), NSF-11 for IgG2a (anti-substance P), G1–5 for IgG2b (anti-G monomer of AChE), NSF-20 for IgG (anti-sub- stance P), and NSF-45 for IgM (antibody P). On completion of washing samples were resuspended in 1 ml of PBS and 10,000 living cells were analyzed in a flow cytometer (Epics XL, Beckman Coulter) equipped with a 488-nm argon laser, previously calibrated with flow check beads (Beckman Coulter).

For experiments aiming to studying the effect of the antibodies on the amount of cell-surface PrPc, infected and non-infected N2a cells plated in 6-well plates (2 \( \times 10^5 \) cells/well) were incubated for 3 days in the presence or absence of a 10 \( \mu g/ml \) concentration of purified mAbs. At the end of this period cells were washed and collected, and 5 \( \times 10^5 \) cells were reacted with a mAb labeled with fluorescein and presenting a binding compatible with the antibody used for the treatment (either with fluorescein-conjugated rabbit polyclonal SAF34 or a mAb reacts for 20 min at room temperature in the dark).

Sample data were acquired under control with a computer equipped with software (System 2, Beckman Coulter). For each sample studied at least 10,000 living cells were analyzed by appropriate gating to exclude dead cells and debris. All fluorescence data were collected on logarithmic scales.

Detection of Intracellular Antibodies Using Immunocytofluorescence—N2a58 cells were cultured on Lab-Tek® chamber slides (Nunc) for 3 days in the presence or absence of a 10 \( \mu g/ml \) concentration of purified mAbs. For intracellular staining of mAbs, cells were rinsed in PBS and fixed with Cytofix (BD Biosciences) at 4 °C for 20 min. After cell-surface saturation with 100 \( \mu g/ml \) goat anti-mouse IgG or IgM immunoglobulin in PBS containing 0.5% bovine serum albumin for 45 min at room temperature, N2a58 cells were permeabilized with 10% Cytoperm (BD Biosciences) in distilled water for 15 min at room temperature and blocked with 0.5% bovine serum albumin in PBS. Cells were then reacted for 45 min at room temperature with phycoerythrin-conjugated goat anti-mouse IgG or IgM immunoglobulin diluted 1/100 in PBS, 0.5% bovine serum albumin. After washing three times with PBS, coverslips were mounted on plastic slides, and staining was visualized with a fluorescence microscope (Olympus IX71).

RESULTS

Screening of 145 Anti-PrP Monoclonal Antibodies for Their Capacity to Inhibit PrPSc Replication in N2a Cells and Concentration Dependence of PrPSc Inhibition—Because the large panel of anti-PrP monoclonal antibodies produced in our laboratory has not yet been tested for its capacity to inhibit PrPSc replication, screening was performed in two cell culture models of transmissible spongiform encephalopathies, a mouse neuroblastoma cell line (N2a) infected with murine PrPSc and a rabbit epithelial cell line (Rov9) infected with ovine PrPSc. Here, we will only fully present results obtained on N2a cells. Results on Rov9 cells were very similar and are summarized under “Results.”

The 145 monoclonal antibodies tested were from 7 different immunization campaigns of KO mice with various immunogens; either PrP peptides, recombinant PrP, or SAFs (see the legend of Table I). They were categorized into seven different families (Table I) depending on the nature of their epitope. Their main characteristics (immunogen, epitope, and species specificity) are shown in Table I. Most of these antibodies were clearly identified binding PrPc or denatured PrP. Some were also shown specifically to immunoprecipitate SAFs (40), but none was shown to bind PrPSc specifically.

This screening was carried out by incubating prion-infected cells with purified anti-PrP monoclonal antibodies at a concentration of 10 \( \mu g/ml \) until they reached confluence (3 days for N2a cells, 1 week for Rov9 cells). During the time-course of this experiment, no cytotoxic effect of antibody was observed. To avoid interference with bovine PrP present in the cell culture medium, the fetal calf serum was depleted of bovine PrP (see “Materials and Methods”). At the end of this 3- or 7-day period cells were collected, and the PrPSc cellular content was determined and normalized on the basis of cell protein content, as a measure of cell number. The first step of PrPSc determination consisted in a rapid purification based on PK treatment, concentration by centrifugation, and denaturation according to the purification method originally developed at Commissariat à l’Energie Atomique (Saclay, France) and now commercialized by Bio-Rad (TeSeE® purification kit, see “Materials and Methods”). During the second step, the denatured PrPSc was detected by means of an appropriate sandwich immunocytoassay (see “Materials and Methods”). Anti-prion activity was expressed as an average percent reduction of PrPSc compared with untreated control cells. Cells treated with irrelevant antibody...
A. Monoclonal anti-PrP antibodies directed against PrP or SAFs were used for the screening. Antibodies of the Pri series were raised against synthetic peptides of human PrP (38). Antibodies of the DPZ series (11C6, 12F10, 4P2, 8C38) were raised against human recombinant PrP (52, 53). Antibodies of the SAF series were raised against proteinase K-treated and formic acid-denatured scrapie-associated fibrils from Syrian hamster-infected brain (263K) (38). Antibodies of the BAR series were raised against ovine recombinant PrP. Antibodies of the SI series were raised against a mutated form of murine PrP (23–231), obtained by heterologous expression in bacteria (54). The far UV CD analysis of this mutated protein (pH 7.0, no added denaturants) revealed an extensive $\beta$-sheet conformation, with little or no $\alpha$-helix present. Antibodies of the $\beta$ series were raised against a recombinant $\beta$-folded human PrP. Antibodies of the Sha series were raised against proteinase K-treated and non-denatured scrapie-associated fibrils from Syrian hamster-infected brain (263K). Linear epitopes recognized by the different mAbs were identified as described under "Materials and Methods." mAbs for which no linear epitope was identified were categorized as "conformational" or "unidentified," indicating that they bind a conformational epitope of PrP or another unknown antigen. Species specificity of the different mAbs was determined by Western blot, two-site immunometric assay, solid phase immobilized epitope immunoassay (SPIE-IA), and/or immunoprecipitation experiments.

B. Monoclonal antibodies of specificity unrelated to PrP. Antibodies of the $\alpha$LI series were raised against $\alpha$-interleukin. Antibodies of the NSP series were raised against the N-terminal part of the substance P peptide coupled to keyhole limpet hemocyanin (KLH) (55). Antibodies of the GI family were raised against the monomer of acetylcholinesterase from Bungarus snake.

### Table I

| Monoclonal antibody | Linear epitope on PrP (when identified) | Species recognized |
|---------------------|----------------------------------------|-------------------|
|                     |                         | Murine PrP | Other PrP |
| A                   | Recombinant ovine PrP | - | - |
| B                   | Protease-K treated, non-denatured SAF from Syrian hamster | - | - |
| C                   | Synthetic peptide Pr3, human PrP | - | - |
| D                   | Recombinant ovine PrP | - | - |
| E                   | Recombinant ovine PrP | - | - |
| F                   | Synthetic peptide Pr3, human PrP | - | - |
| G                   | Recombinant S-folded murine PrP | - | - |
|                     | Recombinant S-folded human PrP | - | - |

### Additional Information

- **Screening mAbs for TSE Therapeutic Effect**

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Screening of mAbs for TSE Therapeutic Effect

Correlation between Cleaning Effect and Cell-surface Binding for N2a Cells—We also tested the cell-surface PrP binding capacity of each antibody by using flow cytometry measurements on infected and non-infected N2a cells (cell-surface indirect pre-staining, Fig. 4A). For every mAb, the staining intensity was equivalent for infected and non-infected N2a cells (isotyp for Rov9 cells), in agreement with the fact that none of these mAbs is known to bind PrPSc.

There was globally an excellent correlation between, on the one hand, antibodies able to cure the infected cells and, on the other hand, those binding cell-surface PrPc efficiently (30 antibodies) (see Fig. 4B). These results are in agreement with what we already knew about these antibodies on the basis of Western blot, solid phase immobilized epitope immunoassay (SPE-IA), ELISA, or immunoprecipitation techniques. Twenty-three antibodies present an intermediary behavior with a low binding capacity associated with little or no cleaning effect. This is not surprising taking into account that both the cell binding and the cleaning effect depend on mAb concentration.

However, there are two exceptions of antibodies, BAR224 and βH3, having no cleaning effect even when used at high concentrations, whereas they clearly bind cell-surface PrPc (see Fig. 4). In addition, two antibodies (BAR232 and βS18, both of IgM isotype) had a low but significant cleaning effect in the absence of cell-surface binding.

Similar Results Were Obtained with Rov9 Cells—The same screening of antibodies (except antibodies of the βH series) was performed on infected rabbit epithelial Rov9 cells constitutively expressing ovine PrPSc. The results were very similar with some divergences due to the species specificity of mAbs.

The same 9 antibodies directed against the PrP octa-repeat region (SAF15, SAF32, SAF33, SAF34, SAF35, SAF37, 4P2, and 3B5) were among the most effective ones, considerably reducing PrPSc levels up to 85% after 1 week of incubation with a 10 μg/ml antibody concentration. Concerning the antibodies directed against the central region of PrP (126–164), four inhibited ovine PrPSc replication up to 70%: BAR221, BAR233, BAR234, and Shα31. SAF83 had no cleaning effect, in agreement with the fact that it does bind ovine PrP poorly (very low cell-surface binding on Rov9 cells was observed by flow cytometry measurements). Surprisingly, SAF53 and SAF61, although they clearly bind cell-surface ovine PrPc on Rov9 cells, had no cleaning effect. All conformational antibodies with a cleaning effect on N2a22L cells, except for 11C6 and βS23, also reduced ovine PrPSc levels on infected Rov9 cells but with significantly lower efficiency (around 10–20% reduction in PrPSc content compared with untreated infected cells).

Within the range of antibody concentration used, we observed a clear dependence of the cleaning effect on the mAb concentration.

Correlation between Cleaning Effect and Cell-surface Binding for N2a Cells

(aLIL–101) showed no detectable reduction in PrPSc levels. We ensured that antibodies added to the culture medium and bound to cellular PrP in vitro did not spuriously block the detecting antibodies in our immunoassay (data not shown).

This screening identified 37 monoclonal antibodies that were efficient at reducing the infected N2a22L cells of their PrPSc content by more than 20% after 3 days of incubation. Those that bind an identified linear epitope are listed in Fig. 1.

Among these antibodies, nine are directed against the octa-repeat region of PrP (Fig. 2A). At a 10 μg/ml concentration, all these anti-octa-repeat antibodies seem to have about the same efficiency (excepting 3B5), dramatically reducing cell PrPSc content by up to 70%.

Eight antibodies directed against the central region of PrP (126–164) also reduced PrPSc levels by up to 90% (Fig. 2B). In addition, 20 other antibodies recognizing a conformational or unidentified epitope inhibited PrPSc formation with varying efficiencies (Fig. 2C). All the other antibodies had no significant effect on PrPSc accumulation (between 80 and 130% of PrPSc in cells compared with untreated infected cells).

Because these data unambiguously demonstrated that anti-PrP monoclonal antibodies could inhibit PrPSc accumulation in N2a infected cells, we performed concentration-dependent experiments (see “Materials and Methods”) to compare inhibition efficiency of the most powerful mAbs. Prion-infected N2a22L cells were cultured for 3 days with various concentrations of selected antibodies ranging from 1 ng/ml to 50 μg/ml. A concentration-dependent inhibition was observed with 50% inhibitory concentration (IC50) ranging from 0.1 μg/ml (0.7 nM) to more than 50 μg/ml (335 nM) (Table II and Fig. 3) without any cytotoxicity observed within the range of antibody concentration used. It is worth noting that, whatever the antibody and concentration used, the cleaning of infected cells was incomplete after 3 days of contact with antibodies. The IC50 measured for SAF34 (1 μg/ml, see Fig. 3) was in agreement with the value of 1.3 μg/ml reported by Perrier et al. (33). In contrast, the IC50 of SAF61 determined here (2.5 μg/ml, see Fig. 5) was slightly higher than previously found by the same author (0.8 μg/ml) (33). Two mAbs, Sha31 and SAF83, directed against the central region of PrP (126–164), seemed particularly efficient, with respective IC50 values of 100 ng/ml (0.7 nM) and 250 ng/ml (1.7 nM) (Fig. 3). The other antibodies recognizing this same central epitope, BAR221, BAR233, SAF61, and BAR215, were less efficient, with IC50 ranging from 600 ng/ml (4 nM) to >50 μg/ml (>335 nM). Surprisingly SAF60 and BAR224, which bind the same epitope, had no significant effect even at high concentrations (Table II). Antibodies recognizing conformational epitopes showed very different IC50 values, ranging from very low concentrations of 100 ng/ml (0.7 nM) for BAR236 to 10 μg/ml (66.7 nM) for BAR232 (Fig. 3 and Table II).

As expected, the control antibody αLIL–101 had no effect on PrPSc levels even when used at high concentrations.  

C. Féraudet, N. Morel, S. Simon, H. Volland, Y. Frobert, C. Créminon, D. Villette, S. Lehmann, and J. Grassi, unpublished results.
face PrPc binding. Again, there were some exceptions of antibodies having no cleaning effect for a 7-day treatment with a 10 μg/ml concentration of mAbs, whereas they clearly bound cell-surface PrPc (11C6, SAF53, SAF61, βS23, and BAR236). Once again, BAR224 clearly bound cell-surface PrPc on infected and non-infected Rov9 cells but had little cleaning effect (around 15% inhibition).

FIG. 2. Results of the screening of mAbs on scrapie-infected neuroblastoma N2a cells: 37 anti-PrP antibodies inhibit PrPSc replication. Nine of the inhibitory mAbs were directed against the octa-repeat region 59–89 (A), 9 against the central region of PrP 130–160 (B), and 20 against a conformational or unidentified epitope of PrP (C). PrPSc levels in infected cells were measured by a two-site immunometric assay (11C6 as the capture antibody and SAF83 AchE as the tracer antibody) after 3 days of culture in the presence of a 10 μg/ml concentration of purified mAbs. Samples of equal protein amount and volume were digested with PK at a ratio of 10 μg of protease/mg of total protein for 15 min at 37 °C. Results are expressed as an average percent content of PrPSc compared with untreated infected cells (N2a22L, black bars). 0% denotes an undetectable level of PrPSc as in non-infected cells (N2a58, bars on the right). Cells treated with irrelevant antibody aIL1–101 showed no detectable reduction in PrPSc levels (gray bars). Data represent the mean of three independent experiments.

TABLE II
Concentration-dependent inhibition of PrPSc replication in N2a22L cells by purified anti-PrP mAbs

| Monoclonal antibody | IC_{50} (μg/ml) | IC_{50} (nM) |
|---------------------|----------------|-------------|
| Sha31               | 0.1            | 0.7         |
| BAR236              | 0.1            | 0.7         |
| SAF83               | 0.25           | 1.7         |
| BAR214              | 0.25           | 1.7         |
| BAR221              | 0.6            | 4.0         |
| SAF34               | 1              | 6.7         |
| BAR226              | 2              | 13.3        |
| BAR233              | 2              | 13.3        |
| SAF32               | 2.5            | 16.7        |
| SAF61               | 2.5            | 16.7        |
| BAR234              | 3              | 20.0        |
| BSS23               | 4              | 26.7        |
| BAR224              | 7              | 46.7        |
| BAR215              | >50            | >333        |
| BAR232              | 10             | 66.7        |
| SAF60               | —              | —           |
| aIL1–101            | —              | —           |

FIG. 3. Concentration-dependent inhibition of PrPSc replication in N2a22L cells by purified anti-PrP mAbs. Infected cells were incubated with various concentrations of mAbs (1 ng/ml to 50 μg/ml) for 3 days. At confluence, cells were lysed, and samples were digested by PK and analyzed by enzyme immunoassay as described in Fig. 2 and under “Materials and Methods.” Values are given as an average percent, where 100% is equivalent to the PrPSc content in untreated infected N2a22L cells and where 0% represents undetectable levels of PrPSc. Data represent the mean of three independent experiments. As an illustration, inhibition curves obtained with six different mAbs are presented.
Effect of Antibodies on Cell-associated, Cell-surface and Released PrPc: Internalization of Antibodies—To get a better understanding on the effect of antibodies on cells, we analyzed the time-course variation of cell-associated PrPc (as measured in non denaturating conditions) and total cell-associated PrP (as measured in denaturing conditions) as well as PrPc and total PrP released in culture medium after 3 h to 4 days of N2a cell culture in the presence of a 10 μg/ml concentration of either SAF34, SAF83, Sha31, BAR224, SAF60, aL1–101, or in the absence of antibody. We were unable to measure PrPc in cell culture medium possibly due to the small amounts and different biochemical properties (PK resistance, aggregation in presence of detergents, etc.) of such PrPc in cell culture medium.

In the absence of antibody treatment, the cell-associated PrPc level is slightly higher (1.3–1.6-fold) in N2a58 (about 21 ng of PrPc/1 million cells) than in N2a22L cells (about 15 ng of PrPc/1 million cells, results not shown), and total PrPc levels in culture medium is 2 times higher in N2a58 (about 9 ng of PrPc released/1 million cells) than in N2a22L (about 4 ng of PrPc/1 million cells, results not shown). However, the total cell-associated PrP content of N2a22L cells (as shown after a denaturing treatment, see Fig. 5B) is higher due to the presence of PrPc. These data suggest that differences in PrP levels observed between N2a58 and N2a22L are a consequence of PrPc production in infected cells.

As expected, antibodies unable to bind and cure the cells as SAF60 or aL1–101 did not have any effect on cellular PrPc (Fig. 5A), on total cell-associated PrP (Fig. 5B), and on total PrP released in cell culture medium (Fig. 5, C and D). These inefficient mAbs were essentially recovered in the medium as free antibodies (at a concentration always equal to 10 μg/ml during the 4 days of culture, data not shown) and did not complex with secreted murine PrP (data not shown) nor cellular PrP (data not shown) nor enter the cell as free antibodies (Fig. 6).

On the other hand, antibodies with a cleaning effect inhibited PrPSc replication, leading to a progressive lowering of PrPSc levels in the cells (Fig. 5A). They have a significant effect on total cell-associated PrP levels in N2a22L cells (Fig. 5B) as a consequence of PrPSc cleaning. In contrast, there is no apparent effect on the PrPc levels of N2a58 and N2a22L cells (Fig. 5B), assuming that the decrease of total denatured cell-associated PrP in N2a22L cells treated with Sha31, SAF34, or SAF83 (up to 5 ng/1 million cells observed in Fig. 5B) results only from PrPSc clearance (a 5-ng decrease/1 million cells, Fig. 5A). At that stage of our study we did not try to differentiate cell-surface PrP from intracellular PrP.

These active antibodies also induced an increase of PrPc levels recovered in the culture supernatant of infected or non-infected neuroblastoma cells: 1.3- and 2.8-fold increase by Sha31, 1.8- and 3.7-fold increase by SAF34, 3.3- and 9-fold increase by SAF83 for N2a58 and N2a22L cells, respectively, after 3 days of culture (Fig. 5, C and D). We also observed that the increasing recovery of PrPc into the culture medium induced by inhibitory mAbs was concentration-dependent (data not shown). It is worth noting that after 3 days of antibody treatment, PrPc levels recovered in cell culture medium are similar for both N2a58 and N2a22L cells. In fact, these data are in agreement with the simple explanation that cell-associated and released PrPc is lowered in infected cells (N2a22L) due to PrPSc replication and that original levels are restored after antibody treatment.

Active antibodies are detected into the cells (Fig. 6), demonstrating an internalization process. Cell-associated PrP-antibody complexes increased with time to the detriment of free antibodies (data not shown). In cell culture medium significant concentrations of mAb-PrP complexes were also measured, whereas free antibody content remained almost constantly close to 10 μg/ml (data not shown).

The paradoxical antibody BAR224 (binding, but not curing the infected cells) had no significant effect on PrPSc levels in cells (Fig. 5A) nor on total cell-associated PrP (Fig. 5, B and C). It induced a small increase in PrPc recovered into the cell culture medium of N2a22L cells, with no effect on N2a58 cells (Fig. 5, C and D). It formed detectable complexes with PrP in the medium (data not shown), but very little of it internalized into the cells (Fig. 6).

To more precisely address the mechanism by which the antibodies reduced PrPSc production and increased secreted PrP, we measured by flow cytometry the amount of cell-surface PrPc on N2a58 and N2a22L cells after 3 days of culture in the presence or absence of a 10 μg/ml concentration of either Pri308, SAF60, BAR224, BAR221, Sha31, SAF32, or SAF34 (see “Materials and Methods”). We observed that a treatment of N2a58 or N2a22L cells with BAR221, Sha31, and SAF32 antibodies increases PrPc amounts on the extracellular membrane (about a 2-fold increase), whereas control antibodies Pri308 and SAF60 had no effect (see Fig. 7 for N2a22L cells). BAR224 and SAF34 antibodies induced a 1.5-fold increase in the cell-surface PrPc amount. Because the total cell-associated PrPc amount did not change in the presence of antibodies bearing a cleaning effect and the cell-surface PrPc amount increased, it seems very likely that the intracellular content in PrPc is lowered.

Co-treatment Experiments—Co-treatment experiments of infected N2a cells with two antibodies directed against different
epitopes were carried out to determine whether we could achieve a more efficient inhibitory effect by binding two different antibodies to a single PrP molecule.

Pairs of mAbs able to bind simultaneously to the same murine PrP molecule at the surface of N2a cells were identified from our previous experiments in establishing sandwich immunoassays for PrP and confirmed by flow cytometry. Five combinations were selected. Three of them involved two antibodies known to recognize different and distant linear epitopes of PrP: SAF34 + Sha31, SAF34 + SAF61, and SAF34 + SAF83 (SAF34 is directed against the octa-repeats; Sha31, SAF61, and SAF83 are directed against the central region of PrP). In addition, we tested a fourth co-treatment including 11C6 (conformational) and SAF83 because these two antibodies present compatible binding and are used in a two-site immunometric assay of mouse PrP (see "Materials and Methods").

Among these five co-treatments, only one (SAF34 + SAF61) appeared synergistic (Fig. 8 C). Indeed, after 3 days of incubation, cells treated simultaneously with SAF34 and SAF61 underwent a more potent inhibition (when expressed in terms of total concentration of antibody) than cells treated with SAF34 or SAF61 alone, in agreement with previously published results (33). For the four other co-treatments (see Fig. 8A for SAF34/Sha31 and 8B for 11C6/SAF83) there was no synergistic effect in that the mixture of mAbs always induced a lower inhibition than observed with the best antibody alone. Moreover, we observed that the time-course of PrPSc decreases in treated cells was not modified when Sha31 and SAF34 were used simultaneously in comparison with treatments with a single antibody (data not shown).
Long Term Efficiency of PrPSc Inhibition—Because all data show that 3 or 4 days of culture in the presence of inhibitory antibodies are not sufficient to completely clean the infected cells of their PrPSc content, we determined whether it was possible to completely cure the cells. N2a22L cells were independently cultured for 11 days in the presence of a 10 \mu{g}/ml concentration of either Sha31, BAR236, SAF34, SAF83, BAR224, SAF60, and IL1–101 or in the absence of mAb. After 7 days of incubation with Sha31, BAR236, SAF83, or SAF34, PrPSc was reduced to undetectable levels, whereas with BAR224, SAF60, and IL1–101, PrPSc levels remained similar to those observed in untreated infected cells. To ascertain the long term efficiency of the treatment, cells previously incubated with the antibodies for 11 days were further cultured for an additional period of 17 days in the absence of mAb. After 7 days of incubation with Sha31, BAR236, SAF83, or SAF34, PrPSc was reduced to undetectable levels, whereas with BAR224, SAF60, and IL1–101, PrPSc levels remained similar to those observed in untreated infected cells. To ascertain the long term efficiency of the treatment, cells previously incubated with the antibodies for 11 days were further cultured for an additional period of 17 days in the absence of mAb. Cells treated with Sha31, BAR236, SAF83, and SAF34 appear to be completely cured since no reappearance of PrPSc level was observed for 17 days after removal of mAbs as seen using either ELISA or a Western blot technique (Fig. 9). In contrast, BAR224, SAF60, and IL1–101 had no impact on PrPSc levels compared with untreated infected cells (Fig. 9).

Interestingly, if N2a22L cells were treated with these same antibodies for a shorter period of 4 days (treatment previously shown to be insufficient to clean PrPSc completely), PrPScs re-accumulated from 4 days after the removal of mAbs. PrPSc content in these treated cells reaching 75–95% of control values 18 days after the 4-day treatment was stopped (data not shown).

**FIG. 7. Effect of anti-PrP monoclonal antibodies on the amount of cell-surface PrPc.** Results of flow cytometry analysis using fluorescein-labeled SAF34 (A) and Sha31 (B) performed on N2a22L. A, N2a22L cells were cultured for 3 days in the presence of mAbs presenting a compatible binding with SAF34. B, N2a22L cells were cultured for 3 days in the presence of mAbs, presenting compatible binding with Sha31. Data represent the mean of three independent experiments using the procedure described under “Materials and Methods.” FITC, fluorescein isothiocyanate.

**FIG. 8. Co-treatment inhibition of N2a22L cells.** Prion-infected N2a cells were incubated for 3 days in the presence of a total concentration of antibodies ranging from 2 ng/ml to 50 \mu{g}/ml; antibodies were used either alone at a 2 \times concentration or as a mixture of two antibodies, each at a 1 \times concentration (2 \times total concentration). Five co-treatments of cells with two antibodies directed against different epitopes were carried out. A, SAF34 + Sha31; B, 11C6 + SAF83; C, SAF34 + SAF61. PrPSc levels were measured by a two-site immunometric assay as described previously in Fig. 2 and under “Materials and Methods.” Data represent the mean of at least two independent experiments.

**DISCUSSION**

To characterize more precisely the possible therapeutic effect of anti-PrP antibodies, we have screened 145 different monoclonal antibodies (mAbs) produced in our laboratory for their capacity to cure prion-infected cell cultures. This screening was performed on two cell models of TSEs, a mouse neuroblastoma cell line (N2a) and a rabbit epithelial cell line (Rov9) constitutively expressing murine and ovine PrPSc, respectively. The results show that the antibodies reduce PrPSc with varying efficiencies in the two cell lines mainly due to species differences. Moreover, our results confirm in a large series of antibodies that the potent inhibition observed with monoclonal antibodies requires as a first step the binding of the antibodies to cell-surface native PrPc, indicating that targeting PrPc is beneficial for prion therapy. Some of our antibodies show a 10-fold lower IC_{50} than those previously reported in the liter-
an IC50 of 9 nM for a 7-day treatment of ScN2a, whereas here affinity (with the exception of mAbs BAR224 and such an effect is to bind cell-surface PrPc with a significant epitope. In fact, it seems that the only requirement to obtain an effect is not directly linked to the targeting of a particular sequence recognized by Fab R1 and R2 (32).

We identified at least four different linear epitopes mediating the curing effect. 1) The first is the very N-terminal sequence recognized by mAb BAR210 (only effective in Rov9 cells expressing ovine PrP). 2) The second is the octa-repeat region (59–89) against which 10 of the 145 antibodies tested are directed. All (except BAR238, which binds PrPc with a very low affinity) systematically inhibit PrPSc accumulation in the two cell line models in a concentration-dependent manner, with an IC50 around 1 µg/ml (about 10 nM). 3) One mAb (8G8) binds the 97–102 sequence of PrP (the effect was observed only on the Rov9 cell line). The Fab D13 and mAbs ICSM35, ICSM37, ICSM42 described previously as potent inhibitors of PrPSc replication in ScN2a (32) or ScRov9 cells (47), respectively, also recognize this region. 4) Finally, 31 of the 145 mAbs are directed against the central region 126–164, but only 7 reduce PrPSc levels in at least 1 of the 2 cell line models in a concentration-dependent manner in direct relation with their capacity to bind native PrPc at the cell surface. Other anti-PrP antibodies recognizing this same central region have previously been shown to prevent infection and inhibit prion replication: Fab D18 (32), 6H4 (91), ICSM 18, and ICSM 19 (37, 47). In addition, 20 antibodies recognizing a conformational epitope also inhibited PrPSc accumulation in a concentration-dependent manner in at least one of the two prion-infected cell lines, thus suggesting that several other epitopes can mediate the cleaning effect. 5) Last, it is worth noting that a fifth linear epitope was strongly suggesting that the mechanism is epitope-independent and more likely reflects a global effect on the PrP trafficking and/or transconformation process. This is in apparent contradiction with previous reports claiming an intrinsic importance of the epitope recognized by antibodies in their inhibitory potency, prion protein residues 132–140 being of critical importance. We believe that our results are much more significant because they are based on the screening of a larger series of mAbs. However, we do not exclude the possibility that other mechanisms targeting specific epitopes may superimpose on an elementary general effect linked to the binding of a mAb to cell-surface PrP.

Different mechanisms can be envisaged to explain the cleaning of antibodies on infected cells, and these hypotheses are not mutually exclusive. The first hypothesis implies a perturbation of the cellular trafficking of PrPc leading to a lowering of the provision of substrate PrPc to the intracellular compartment where PrPc to PrPSc conversion occurs. Thus, the prevention of PrPc endocytosis from cell surface to endosomes and the sequestration of PrPc on the cell surface by the anti-PrP antibodies and/or the release of PrPc from the plasma membrane to the medium could prevent PrPSc replication. Because the N terminus domain of PrP is essential for PrP endocytosis (48), cellular trafficking (49, 50), and membrane localization (51), it can be hypothesized that anti-N-terminal antibodies (anti-octa-repeat and BAR210) prevent PrPSc replication in this way by impeding or reducing PrP endocytosis. This explanation, however, does not hold necessarily for antibodies directed against downstream epitopes, which appear as efficient.

The data presented in this paper are not sufficient to describe in detail the influence of PrPSc replication on PrP trafficking in infected cells and the details of the mechanism leading to the cleaning of infected cells by antibodies. However, our work clearly demonstrates important changes in PrP metabolism between infected and non-infected cells in the absence or presence of active antibodies. In the absence of antibody treatment, a clear difference is evidenced between

![FIG. 9. Long term elimination of PrPSc from scrapie-infected N2a cells treated with anti-PrP monoclonal antibodies.](http://www.jbc.org/)
N2a58 and N2a22L, with an increased PrPc level in the supernatant of non-infected cells (twice more PrPc) and a lower amount of cell-associated PrPc in infected cells (very likely resulting in PrPSc production). Of course the total amount of PrP is higher in infected cells due to the production of PrPSc. In infected cells, the effect of curing antibodies is to lower cell-associated PrPSc levels, whereas PrPc levels are restored to the level observed in non-infected cells. In both types of cells the effect of antibody binding is to increase the level of PrPc at the cell surface of the cells and to increase the amount of PrPc recovered in the culture medium so that equivalent levels are recovered in supernatants of infected and non-infected cells (Fig. 5D). It is worth noting that the increased amounts of PrPc recovered in culture supernatants due to the antibody treatment may reflect either an increased stability of PrPc in culture medium due to the presence of mAbs or an active process inducing an increased secretion of PrPc. For instance, one can imagine that the binding of mAbs increases the residence time of PrPc at the cell surface (in agreement with our data and a previous report (66)), thus favoring the action of proteases releasing PrPc in the culture medium. Whatever the mechanism underlying this phenomenon, it is worth noting that if the same release is observed in vivo, it could partly explain the curing effect of anti-PrP mAbs as the presence of dimeric soluble PrPc has been shown to be beneficial (26). Taken together, these data strongly support the idea that mAbs interfere with PrPc trafficking and not with a process involving PrPSc. The observation that mAbs with a cleaning effect sequestrate PrPc at the cell surface also strongly supports this idea.

On the other hand, since the cellular level of PrPSc is determined by the rate of its formation from PrPc and its catabolism, PrPSc accumulation could also be reduced due to PrPc degradation induced by antibodies. In this study we were unable to see a significant effect of anti-PrP mAbs as the presence of dimeric soluble PrPc has been shown to be beneficial (26). Taken together, these data strongly support the idea that mAbs interfere with PrPc trafficking and not with a process involving PrPSc. The observation that mAbs with a cleaning effect sequestrate PrPc at the cell surface also strongly supports this idea.

Alternatively, in a second hypothesis, as suggested in previous studies (31, 32), antibodies could inhibit the contact between PrPc and PrPSc and/or a cofactor critical for the conversion by binding to PrPc or PrPSc isoforms. This mAb/PrP interaction could occur on the cell surface or in the endosomal compartments as antibodies can enter the cells (Fig. 6). This effect is perfectly compatible with and possibly additive to the effect observed on trafficking. In our sense this effect should be rather due to PrPc/antibody interaction and not to any kind of PrPSc/antibody binding since none of our antibodies was shown to bind PrPSc (none was shown by flow cytometry measurements to bind more specifically to infected than non-infected cells).

As a third hypothesis, when binding PrPc, antibodies could interfere with PrPc transconformation either at the cell surface or in the intracellular compartment. Once again, this effect is possibly additive to the effect observed on trafficking.

Concerning the paradox of antibodies curing but not binding the infected cells (BAR232 and βS18), it is worth noting that they are both of the IgM isotype. This could interfere with flow-cytometry measurements and explain the apparent absence of binding. The situation is different for the antibodies binding cell-surface PrP but not curing the infected cells (βH3 and BAR224 for N2a cells; 11C6, SAF53, SAF61, βS23, BAR236, and BAR224 for Rov9 cells). The case of BAR224 is interesting in that it also induces an increase of cell-surface PrPc and an increased release of PrPc in the culture medium (as observed with inhibitory antibodies), but it is poorly internalized into the cells. This suggests that this antibody can achieve the first steps of a process leading to the therapeutic effect (binding and sequestration of cell-surface PrPc) but is unable to achieve further intracellular steps where other inhibition mechanisms could take place. This observation supports the second and the third hypotheses detailed above.

A recent work (47) suggests that antibodies targeting both PrPc and PrPSc clean infected Rov9 cells more efficiently than mAbs exclusively binding PrPc, raising the possibility that targeting PrPSc could be crucial for optimal inhibitory potency. We have no data supporting this idea because we think that none of the antibodies tested during this study actually binds PrPSc. Recently (40) we have shown that some of the mAbs used in the present work can efficiently immunoprecipitate both PrPc and PrPSc, whereas others selectively immunoprecipitate aggregated PrPSc as found in a TSE-infected brain. However, we do not believe that these immunoprecipitation techniques can identify antibodies directed against PrPSc but, rather, reflect nonspecific binding between aggregated PrPSc and mAbs immobilized on the polydisperse solid phase. Anyway, we have not seen any correlation between the capacity of an antibody to immunoprecipitate PrPSc and a cleaning effect. For instance, Sha31 and 12F10 are both able to immunoprecipitate PrPc and the PK-digested PrPSc (PrP27–30) with approximately the same efficiency, whereas only Sha31 shows a cleaning effect in Rov9 and N2a22L cells in the conditions tested. Moreover, BAR233, which specifically immunoprecipitates PrPc, has an equivalent or even better cleaning effect and IC50 than SAF61 and 11C6, which are both able to immunoprecipitate PrPc and SAFs. Finally, antibodies specifically immunoprecipitating PrPSc (βS36, Sha52, Sha29, and Sha9, see Morel et al. (40)) were devoid of any cleaning activity.

Co-treatment experiments performed with a mixture of two antibodies compatibly binding cell-surface PrPc did not show any benefit with regard to a treatment involving a single mAb, except for the combination of SAF34 + SAF61, in agreement with a previous study (33). However, even this combination does not appear to be more efficient than the most efficient antibodies used in monotherapy. These data are important and will guide the forthcoming in vivo experiments.

Further work is needed to complete our understanding of the mechanism by which anti-PrP mAbs can clean scrapie-infected cells. This implies a more detailed study of the influence of antibodies on PrP trafficking and metabolism as well as the fate of antibodies at the surface of and inside the cells. These studies are currently being performed in our laboratory and will be described in other reports. However, the final goal is to look at the effect of these mAbs in in vivo situations and first in scrapie-infected mice. This present preliminary study allowed us to identify mAbs seemingly well suited to therapeutic applications and will be a useful guide in designing further animal studies.

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