Immunotherapeutic effect of anti-PrP monoclonal antibodies in transmissible spongiform encephalopathy mouse models: pharmacokinetic and pharmacodynamic analysis

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Prion diseases are transmissible neurodegenerative disorders for which no therapeutic or prophylactic regimens exist. Passive immunization with appropriate antibodies directed against the cellular form of the prion protein (PrPC) can delay the onset of prion disease after peripheral infection, but mechanisms and parameters determining their in vivo efficacy remain unknown. In the present study, we characterized the main pharmacokinetic properties of anti-PrP antibodies in different mouse models expressing various levels of PrPC (Prnp0/0, C57BL/6 and tga20 mice) in correlation with therapeutic effect. Plasma levels of free antibodies, total endogenous PrPC and PrPC–antibody complexes were monitored after a single intraperitoneal monoclonal antibody (mAb) injection. Efficacy in delaying PrPSc peripheral accumulation seemed to be associated with mAb capacity to form long-lasting complexes with endogenous PrPC in the plasma. In agreement with previous observations on cellular models of transmissible spongiform encephalopathy infection, we observed that injection of anti-PrP antibodies induced a large (up to 100-fold) increase in circulating PrPC. Finally, the most efficient antibody extended the lifespan of infected animals greatly. These results allowed us to define critical characteristics of anti-PrP mAbs associated with therapeutic efficacy and could constitute a useful reference for designing optimized passive immunotherapies for prion diseases.

INTRODUCTION

Transmissible spongiform encephalopathies (TSEs) (Aguzzi & Polymenidou, 2004; Collins et al., 2004) are fatal, neurodegenerative disorders that include Creutzfeldt–Jakob disease (CJD) in humans, scrapie in sheep and bovine spongiform encephalopathy (BSE) in cattle. The disease is characterized by a post-translational transconformation of the host-encoded prion protein (PrPC), which is rich in α-helical regions and sensitive to proteinase K (PK), into a partially PK-resistant and β-sheet-rich isoform named PrPSc, accumulating in tissues (Prusiner, 1998). According to the ‘protein-only hypothesis’, PrPSc itself is infectious (Prusiner, 1982).

No effective prophylactic or therapeutic treatment is currently available. With the emergence of variant CJD (v-CJD) disease linked to the BSE epizootic in cattle (Hill et al., 1997; Will et al., 1996) and the later identification of v-CJD inter-human transmission by blood transfusion (Llewelyn et al., 2004; Peden et al., 2004; Wroe et al., 2006), the development of therapeutic strategies has become a priority.

In v-CJD, contamination occurs by the peripheral route and infectious agent accumulates in peripheral tissues long before neuroinvasion. Therefore, compounds impairing prion dissemination in peripheral tissues could be used as therapeutic agents.

Given the few possibilities presently offered by drugs (Haik et al., 2004; Irani et al., 2003; Otto et al., 2004; Weissmann & Aguzzi, 2005; Whittle et al., 2006), research has evaluated new therapeutic approaches (Mallucci & Collinge, 2005),
including immunological strategies (Aguzzi & Sigurdson, 2004; Trevitt & Collinge, 2006).

Anti-PrP antibodies are able to cure prion-infected cells (Beringue et al., 2004; Cardinale et al., 2005; Enari et al., 2001; Feraudet et al., 2005; Kim et al., 2004; Peretz et al., 2001; Perrier et al., 2004). In vivo, using peripheral TSE infection animal models, continuous intraperitoneal (i.p.) treatment with anti-PrP antibodies delays disease development, with variable efficacy (Sigurdsson et al., 2003; White et al., 2003). In a similar mouse model, the passive transfer of anti-PrP antibodies into the central nervous system has strong adverse effects (Solforosi et al., 2004).

In this study, 13 anti-PrP antibodies, selected for their in vitro properties in permanently infected cell models (Feraudet et al., 2005), were investigated for their (i) therapeutic effects and (ii) pharmacokinetic and pharmacodynamic properties in different TSE mouse models.

**RESULTS**

**Treatment with anti-PrP antibodies delays peripheral PrPSc dissemination**

C57BL/6 and tga20 mice inoculated i.p. with ME7 were treated i.p. with 1 mg antibody (Table 1) three times at

| Table 1. mAb characteristics and pharmacokinetic data

Linear epitopes recognized by the different mAbs were identified as described by Morel et al. (2004). Antibodies for which no linear epitope was identified are categorized as ‘unidentified’, indicating that they bind a conformational epitope of PrP or another unknown antigen. In vitro $IC_{50}$ was determined in N2a22L cells after 3 days culture in the presence of antibodies (Feraudet et al., 2005). –, No effect on PrPSc levels even when using high concentration of mAbs; MRT, mean residence time; ND, not determined; NM, not measurable; $V_{ss}$, apparent steady-state volume of distribution.

<table>
<thead>
<tr>
<th>mAb</th>
<th>Isotype</th>
<th>Linear epitope on PrP (when identified)</th>
<th>C57BL/6</th>
<th>tga20</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>mAb–PrP complexes</td>
<td>Plasma PrP</td>
<td>$V_{ss}$</td>
</tr>
<tr>
<td>BAR223</td>
<td>IgG2α*</td>
<td>Unidentified</td>
<td>7.3 ± 0.6</td>
<td>619 ± 98</td>
</tr>
<tr>
<td>BAR233</td>
<td>IgG2b*</td>
<td>141-FGS YD E R Y R R-151</td>
<td>2.6 ± 0.7</td>
<td>1030 ± 111</td>
</tr>
<tr>
<td>BAR236</td>
<td>IgG2α*</td>
<td>Unidentified</td>
<td>4.1 ± 1.1</td>
<td>1662 ± 217</td>
</tr>
<tr>
<td>SAF61</td>
<td>IgG2α†</td>
<td>142-GS DYEDRYRREN-153</td>
<td>1.4 ± 0.1</td>
<td>537 ± 156</td>
</tr>
<tr>
<td>BAR226</td>
<td>IgG1*</td>
<td>Unidentified</td>
<td>2.5 ± 0.2</td>
<td>1600 ± 454</td>
</tr>
<tr>
<td>Sha31</td>
<td>IgG1§</td>
<td>145- E YD R Y R E-152</td>
<td>3.1 ± 0.5</td>
<td>0 ± 0</td>
</tr>
<tr>
<td>βS23</td>
<td>IgG2α§</td>
<td>Unidentified</td>
<td>2.3 ± 0.1</td>
<td>638 ± 58</td>
</tr>
<tr>
<td>11C6</td>
<td>IgG2all</td>
<td>Unidentified</td>
<td>1.6 ± 0.4</td>
<td>812 ± 58</td>
</tr>
<tr>
<td>BAR214</td>
<td>IgG2α*</td>
<td>Unidentified</td>
<td>4.2 ± 0.1</td>
<td>1057 ± 28</td>
</tr>
<tr>
<td>BAR221</td>
<td>IgG1*</td>
<td>141-FGS DYEDRYRREN-151</td>
<td>1.5 ± 1.3</td>
<td>368 ± 57</td>
</tr>
<tr>
<td>SAF83</td>
<td>IgG1†</td>
<td>Recognize solid-phase immobilized peptide 126–164</td>
<td>0.4 ± 0.2</td>
<td>126 ± 1</td>
</tr>
</tbody>
</table>

*Antibodies of the BAR series were raised against ovine recombinant PrP.
†Antibodies of the SAF series were raised against PK-treated and formic acid-denatured scrapie-associated fibrils from Syrian hamster-infected brain (263K) (Demart et al., 1999).
§Antibodies of the Sha series were raised against PK-treated and non-denatured scrapie-associated fibrils from Syrian hamster-infected brain (263K).
$Antibodies of the βS series were raised against a mutated form of murine PrP (23–231), obtained by heterologous expression in bacteria (Meggio et al., 2000). Far-UV circular dichroism analysis of this mutated protein (pH 7.0, no added denaturants) revealed an extensive β-sheet conformation, with little or no α-helix present.
∥Antibody 11C6 was raised against human recombinant PrP.
¶Antibody Pri308 was raised against a synthetic peptide of human PrP: KTNMKHMAGAAAGAVGGGLG (Demart et al., 1999).
#Antibody NSP35-6 was raised against the N-terminal part of the substance P peptide coupled to keyhole limpet haemocyanin.
**Antibody AMYL-2 was raised against the N-terminal part of the amyloid peptide (Alzheimer).
1 week intervals, starting at 7 days post-inoculation (p.i.). Groups of animals were sacrificed at 28 and 56 days p.i., and peripheral PrPSc spread was measured by ELISA and immunohistochemistry.

At 28 days p.i., in the untreated and mock-treated (Pri308 control antibody) groups, PrPSc was observed in most spleen lymphoid follicles (respectively 77.9 ± 13.0 % and 85.9 ± 5.5 % in C57BL/6 mice; Fig. 1a) and high splenic PrPSc levels were measured [Fig. 1(b) for C57BL/6 and Fig. 1(d) for tga20 mice]. Only a few lymphoid follicles were positive in Peyer patches and popliteal lymph nodes (Fig. 1c).

In treated mice, a strong effect was observed with some monoclonal antibodies (mAbs). A first group of mAbs (BAR223, BAR233, BAR236, SAF61, BAR226, Sha31, βS23, BAR214, 11C6 and BAR221) reduced PrPSc splenic

**Fig. 1.** Short treatment with anti-PrP antibodies delays peripheral PrPSc spread after i.p. inoculation of C57BL/6 and tga20 mice. Anti-PrP antibody treatment consisted of three i.p. injections of 1 mg at 7, 14 and 21 days p.i. performed in C57BL/6 (a–c) and tga20 (d) mice inoculated i.p. with ME7. In (a) and (b), C57BL/6 mice were sacrificed at either 28 days p.i. (white bars) or 56 days p.i. (grey bars). Splenic PrPSc levels were quantified by ELISA (b) or by measuring the percentage of positive follicles by immunohistochemistry (a) (between 20 and 60 follicles counted per spleen; see Methods). Each group consisted of six or seven animals. In (c), PrPSc levels in Peyer patches were assessed by measuring the percentage of positive follicles by immunohistochemistry (see Methods) in each individual mouse (○). Mean percentages (bars) were calculated, accounting for and pooling total follicles detected in each group of seven mice [mean, 15 follicles counted per group (minimum, 11; maximum, 20)]. In (d), groups of six tga20 mice were sacrificed at 28 days p.i. and splenic PrPSc levels were measured by ELISA (see Methods). Each box plot identifies the middle 50 % of the data, the median and the extremes. The dashed line represents the cut-off, calculated as mean +3 SD using data from uninoculated mice. Controls included inoculated and uninoculated mice untreated or treated with Pri308 control antibody. S*, P<0.05; S**, P<0.01; S***, P<0.001.
accumulation in both C57BL/6 and tga20 mice, with various efficacies (Fig. 1). At least half of the mice treated with BAR223, BAR233 and BAR236 harboured PrPSc levels under the detection threshold (Fig. 1b, d), and the number of positive follicles was reduced dramatically in comparison with controls (Fig. 1a). In parallel, treatment with other mAbs, like 11C6 or BAR221, had a significant but limited effect on both PrPSc splenic accumulation (Fig. 1b, d) and number of positive lymphoid follicles (Fig. 1a).

A second group of mAbs (SAF83, βS12 and SAF34) had no significant effect either on splenic PrPSc amount (Fig. 1b, d) or on lymphoid follicle involvement (approx. 47.0 ± 15.0 % of positive follicles; Fig. 1a).

At 56 days p.i., PrPSc accumulation levels and follicular accumulation in spleen were similar in treated and untreated animals (Fig. 1a, b). However, in mice treated with the antibodies exhibiting the strongest effect at 28 days p.i. (BAR223, BAR233 and BAR236), PrPSc follicular accumulation in Peyer patches (Fig. 1c) and popliteal lymph nodes (data not shown) was reduced significantly in comparison with controls. In contrast, SAF83 and SAF34 antibodies had no effect on either PrPSc accumulation (Fig. 1a, b) or PrPSc dissemination in Peyer patches (Fig. 1c).

Taken together, these data demonstrate that some anti-PrP mAbs can delay PrPSc accumulation and dissemination in the lymphoreticular system.

**Delay in peripheral PrPSc dissemination is associated with a lengthening of survival time**

To evaluate a possible effect on survival time, C57BL/6 mice were inoculated i.p. and treated with mAb BAR236 (1 mg i.p. injections), one of the most efficient mAbs (see above). BAR236 treatment induced very significant lengthening of the incubation period (survival time of 361.2 ± 37.8 days) compared with untreated controls (248.3 ± 6.7 days) and Pri308-treated controls (274.3 ± 10.3 days), corresponding to a delay of 45.5 and 31.7 %, respectively (Fig. 2).

In tga20 mice, BAR236 treatment resulted in a limited but statistically significant lengthening of incubation period (6.7 % delay; S***, P ≤ 0.01) by comparison with untreated controls; 4.2 % delay: S*, P ≤ 0.05 in comparison with Pri308-treated controls; Fig. 2).

**Anti-PrP antibody pharmacokinetic and therapeutic efficacy in C57BL/6 mice**

To understand differences in therapeutic effect in vivo between mAbs and divergence between in vivo and in vitro inhibition effects (Table 1 cf. Fig. 1), we measured the variation in plasma levels of (i) free antibodies, (ii) total PrP and (iii) PrP–antibody complexes, after a single 1 mg i.p. administration of anti-PrP mAbs to uninoculated wild-type mice. As controls, we checked that C57BL/6 mice treated with PBS showed no change in plasma levels of PrPC (20 ng ml⁻¹), mAb–PrP complex or anti-PrP antibody (data not shown).

For AMYL-2, NSP35-6 and Pri308 mAbs (respectively IgG2a, IgG2b and IgG1), which do not recognize native murine PrPC, no change in plasma PrPC levels (20 ng ml⁻¹) (Fig. 3k) and no presence of PrP–mAb complex (Fig. 3l) were detected. Plasma concentrations of antibodies decreased slowly (half-lives of 7.4 ± 0.4 days and 5.9 ± 1.0 days for NSP35-6 and Pri308, respectively; Fig. 3j), with an apparent volume of distribution (Vss) close to 3 ml (Table 1).

Administration of mAbs recognizing murine PrPC induced (i) a rapid appearance of PrP–antibody complexes, (ii) a rapid diminution of free circulating antibodies and (iii) a large increase in plasma PrPC levels (Fig. 3a–i). The amplitude and dynamics of these changes varied greatly between mAbs, which could be split into three groups, without any correlation with mAb isotype.

For group A mAbs (SAF34, SAF83, βS12 and BAR221), free and bound antibodies were only detectable shortly after
administration (maximum 2 days, mean $t_{1/2} < 2$ days; Fig. 3g, i). This short residence time was associated with a small increase in plasma PrPC levels (5- to 20-fold; Fig. 3h; Table 1). The large $V_\infty$ (Table 1) suggests that these mAbs might be trapped rapidly in tissues.

The group B mAbs (BAR233, SAF61, BAR226, $\beta$S23, 11C6 and Sha31) remained detectable up to 7 days post-injection and the $V_\infty$ was reduced (Table 1; Fig. 3d, f). Free antibodies were replaced rapidly in plasma (mean $t_{1/2}$ of 1.0 ± 0.3 days) by PrP–antibody complexes. Complexes reached maximal levels at about 2 days, but disappeared rapidly from the circulation (mean $t_{1/2}$ of 1.4 ± 0.5 days and mean residence time of 2.3 ± 0.6 days; Fig. 3f; Table 1). A marked increase in plasma PrPC levels was observed (35- to 85-fold; Fig. 3e).

The group C mAbs (BAR223, BAR236 and BAR214) were characterized by detectable levels of circulating (free or bound) mAbs up to 10 days post-injection (Fig. 3a, c) and reduced $V_\infty$ (Table 1). Free antibodies disappeared rapidly (mean $t_{1/2}$ of 1.3 ± 0.3 days), whilst complexes increased progressively to levels at between 4 and 7 days before progressive disappearance (mean $t_{1/2}$ of 1.7 ± 0.4 days and mean residence time of 5.2 ± 1.8 days; Fig. 3c; Table 1). Moreover, a major increase in total plasma PrPC levels was observed (from 35- to 100-fold; Fig. 3b).

Interestingly, whilst group A antibody treatment induced no or very poor therapeutic effect, treatments with group B and C antibodies were respectively responsible for a moderate to large delay in accumulation of PrPSc in peripheral tissues and clinical onset in C57BL/6 mice. These observations suggest that the longer the half-life and mean residence time of PrP–antibody complexes in plasma, the greater the therapeutic effect of the antibody.

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**Fig. 3.** Time-course variation of free anti-PrP antibodies, PrP and PrP–antibody complexes in Prnp<sup>−/−</sup>, C57BL/6 and tga20 mouse plasma after a single i.p. antibody injection. Prnp<sup>−/−</sup> ( ), C57BL/6 ( ) and tga20 ( ) mice were injected i.p. with 1 mg BAR236 (a–c), $\beta$S23 (d–f), SAF34 (g–i) or Pri308 (j–l) antibody and bled before injection (day 0) and at regular time points post-injection (6 h and 2, 4, 7, 10, 15 and 21 days). Free antibodies (a, d, g, j), PrPC (b, e, h, k) and PrP–antibody complexes (c, f, i, l) were measured in mouse plasma (see Methods). Data represent the mean of groups of three animals. The pharmacokinetic profile of BAR236 is representative of those obtained for group C anti-PrP antibodies in C57BL/6 mice (BAR223, BAR236, BAR214), $\beta$S23 is representative of group B (BAR233, SAF61, BAR226, $\beta$S23, 11C6, Sha31), SAF34 of group A (SAF34, SAF83, $\beta$S12, BAR221) and Pri308 of control antibodies (AMYL-2, NSP35-6, Pri308). GAM, Goat anti-mouse antibody.
Impact of PrPC on pharmacokinetics of anti-PrP antibodies

In tga20 mice, treatment efficacy remained poor and only two mAbs prolonged survival times slightly: BAR214 and BAR236 (Fig. 2; Table 2). This suggests that PrPC expression could have a dramatic impact on the efficacy of passive immunotherapy. *Prnp*<sup>0/0</sup>, C57BL/6 and tga20 mice were thus studied after administration of 1 mg BAR236 (group C), βS23 (group B), SAF34 and SAF83 (group A), Pri308 (control mAb) or PBS.

In *Prnp*<sup>0/0</sup> mice, all mAbs had similar profiles (data not shown), with no detectable PrP or antibody–PrP complex. After peaking shortly after i.p. mAb administration, free antibodies were eliminated progressively from plasma (mean *t<sub>1/2</sub>* of 9.8 ± 3.0 days), suggesting that all antibodies are absorbed rapidly at the peritoneal level into the blood circulation. A greatly shortened plasma half-life was observed for mAb SAF83 (*t<sub>1/2</sub>* = 3.4 ± 0.1 days), suggesting that this particular antibody is unstable, thus accounting for its lack of *in vivo* therapeutic effect.

In tga20 mice, administration of the same mAbs induced a phenomenon similar to that observed in C57BL/6 mice. However, the course of events was greatly modified, with:

- (i) a much-reduced half-life of free mAbs (Fig. 3a, d);
- (ii) a more rapid (*T<sub>max</sub>* of 6 h versus 2–4 days in C57BL/6 mice) and massive (*C<sub>max</sub>* from 2- to 6-fold higher) appearance of antibody–PrP complexes, but with shorter residence time of complex (reduced by 70% to 90%) (Fig. 3c, f, i);
- (iii) a transient (*T<sub>max</sub>* of 6 h versus 2–4 days in C57BL/6 mice) and massive (*C<sub>max</sub>* from 2- to 6-fold higher) increase of total plasma PrPC levels (Fig. 3b, e, h).

Interestingly, only small differences were observed between the profiles of group A, B and C antibodies in this model. As no difference could be observed between the three mouse models with antibodies irrelevant to PrP or not binding to native murine PrPC (Pri308; Fig. 3j), alterations observed in the antibodies’ pharmacokinetic properties can be attributed mainly to PrP expression levels [even if an effect of the genetic background cannot be excluded, as tga20 and *Prnp*<sup>0/0</sup> transgenic mice possess half of the C57BL/6 genetic background (129Sv × C57BL/6)].

### Table 2. Survival times of tga20 mice after a short anti-PrP antibody treatment

<table>
<thead>
<tr>
<th>Inoculation</th>
<th>Anti-PrP mAb</th>
<th>Survival time*</th>
<th>Delay†</th>
<th>Statistical significance‡</th>
</tr>
</thead>
<tbody>
<tr>
<td>–</td>
<td>None</td>
<td>Alive (6)</td>
<td>&gt;250</td>
<td>NA</td>
</tr>
<tr>
<td>+</td>
<td>None</td>
<td>Alive (6)</td>
<td>&gt;250</td>
<td>NA</td>
</tr>
<tr>
<td>+</td>
<td>Pri308</td>
<td>127.2 ± 2.9 (6) NA</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>+</td>
<td>Pri308</td>
<td>130.2 ± 2.3 (5) 3.0 (NA)</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>+</td>
<td>BAR236</td>
<td>135.7 ± 1.7 (6) 8.5 (5.5)</td>
<td><em>P</em> = 0.001</td>
<td>(<em>P</em> = 0.017)</td>
</tr>
<tr>
<td>+</td>
<td>BAR214</td>
<td>135.2 ± 3.3 (5) 8.0 (5.0)</td>
<td><em>P</em> = 0.009</td>
<td>(<em>P</em> = 0.056)</td>
</tr>
<tr>
<td>+</td>
<td>11C6</td>
<td>132.5 ± 2.5 (6) 5.3 (2.3)</td>
<td><em>P</em> = 0.017 (NS)</td>
<td>NA</td>
</tr>
<tr>
<td>+</td>
<td>SAF34</td>
<td>132.0 ± 3.8 (4) 4.8 (1.8)</td>
<td><em>P</em> = 0.01 (NS)</td>
<td>NA</td>
</tr>
<tr>
<td>+</td>
<td>BAR226</td>
<td>131.2 ± 4.4 (6) 4.0 (1.0)</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>+</td>
<td>Shal1</td>
<td>130.2 ± 2.3 (5) 3.0 (0.0)</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>+</td>
<td>BAR236</td>
<td>129.8 ± 4.6 (6) 2.6 (−0.4)</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>+</td>
<td>BAR221</td>
<td>129.0 ± 2.1 (5) 1.8 (−1.2)</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>+</td>
<td>SAF61</td>
<td>128.6 ± 1.1 (6) 1.4 (−1.6)</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>+</td>
<td>βS23</td>
<td>128.5 ± 3.5 (6) 1.3 (−1.7)</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>+</td>
<td>SAF34</td>
<td>128.0 ± 3.1 (6) 0.8 (−2.2)</td>
<td>NS</td>
<td>NS</td>
</tr>
</tbody>
</table>

*Survival times are expressed as mean days ± SEM, followed by the number of animals in parentheses.
†Delays (in days) were calculated from data for the inoculated untreated group, followed by the delay calculated from data for the inoculated Pri308-treated group in parentheses. NA, Not applicable.
‡Statistical significance was calculated from data for the inoculated untreated group, followed by the statistical significance calculated from data for the inoculated Pri308-treated group in parentheses. NS, Non-significant (*t*-test).

Biochemical characterization of PrPC in plasma

As anti-PrP antibody administration increased plasma PrPC levels massively, we investigated the nature of this PrPC in C57BL/6 and tga20 mice.

Measurements in protein A-immunoprecipitated plasma fraction revealed that the PrPC increase in treated mouse plasma was mainly (but not exclusively) composed of PrP-antibody complexes in C57BL/6 mice (80% for BAR236; 100% for Sha1; data not shown). The increase in plasma PrPC could be due to enhanced stability of PrPC when binding to anti-PrP mAbs. Alternatively, and not exclusively, it could also be due to release of PrPC molecules from undetermined cells or tissues into the blood.

To characterize the biochemical form of PrPC further, we used different immunoassays to measure (i) full-length PrPC, (ii) full-length and N-terminally truncated PrPC, and (iii) N-terminal free circulating fragments (Fig. 4). Measurements before treatment revealed that both biochemical forms of PrPC (full-length and N-terminally truncated) are present in plasma. Proportions of the different forms did not change significantly after treatment, suggesting that the process leading to increased plasma PrPC levels does not induce a major perturbation in PrP catabolism. A similar experiment was performed on treated tga20 plasma analysed via Western blotting (Fig. 5), showing the increase in PrPC levels consequent to treatment with BAR236 and βS23, but not with Pri308.
Finally, both biochemical forms of PrPC were also identified before and after treatment: N-terminally truncated (visible after Sha31 immunoprecipitation and Sha31 immunoblotting, and mainly after treatment when plasma PrPC levels are higher) and full-length PrPC (visible before and after treatment, with Sha31 and SAF34 immunoprecipitation coupled with Sha31 or SAF32 immunoblotting).

DISCUSSION

Our data reinforce published results on the in vivo efficacy of passive immunotherapy in treating TSEs (Sigurdsson et al., 2003; White et al., 2003). We observed only a moderate increase in lifespan in treated mice, probably because of a short administration protocol limited to the first quarter of the incubation period (50 mg kg$^{-1}$ weekly), whereas in previous work (White et al., 2003), the animals were treated throughout their lives with a similar dose.

Moreover, the prion dose inoculated was quite high (100 µl of 2% inoculum), resulting in 'harsh' conditions to observe mAb therapeutic benefits.

Further, the present results indicate that antibody administration impairs PrPSc accumulation in peripheral tissue, which probably accounts for the treatment-induced delay in disease progression. However, this effect does not persist after discontinuation of the treatment.

These findings suggest that, rather than having a true 'curing effect', in vivo passive immunotherapy might only control prion accumulation temporarily. Because of the potential adverse effects, such a limited effect could compromise the potential value of passive immunotherapy in humans, even if initiated in the early phase of the disease. However, an optimized therapeutic regimen incorporating an alternative delivery route per dose/administration interval, or bioengineered antibodies, could
circumvent this limitation. Such improved treatments are currently under evaluation.

All of the anti-PrP antibodies included in this study had curing properties in prion-infected cell models (Feraudet et al., 2005). However, in vivo, some (group A) did not have any positive effect, whereas others (groups C and B) delayed the course of the disease significantly in conventional mice. This difference did not appear to be linked to antibody isotype, PrP-binding epitope or even affinity, but seemed to be associated with intrinsic antibody pharmacokinetic properties. An apparent positive correlation was observed between treatment efficacy and antibody mean residence time in the plasma compartment, either as free antibodies or as PrP–antibody complexes, even if the number of antibodies tested was not large enough to establish an undoubted relationship. Antibodies with large apparent volumes of distribution and/or short apparent residence time in plasma were, in our experiments, devoid of any beneficial effect.

Large variations in the pharmacokinetics of immunoglobulins have been reported in various models of passive immunotherapy (Lobo et al., 2004), without clear explanations.

Here, antibody administration was performed arbitrarily on a weekly basis, whilst for all antibodies (excluding BAR223), mean residence time was <5 days. This might explain the poor efficacy of mAbs and, consequently, more frequent administrations may lead to a more effective treatment. This point is actually under evaluation.

One of the main and original findings of this study is the demonstration that a major consequence of antibody administration is a massive increase in plasma PrPC concentration, in relation to the appearance of PrPC–antibody complexes. In N2a cells treated with anti-PrP antibodies, a similar effect was observed (albeit of lesser magnitude), with an increase in PrPC levels in cell-culture medium (Feraudet et al., 2005). Two different explanations can be proposed for this increase in plasma PrPC: the release of new PrPC molecules from other compartments, and/or a longer mean half-life (stabilization) of plasma PrPC due to the formation of PrPC–antibody complexes. Several arguments argue strongly against this second hypothesis:

- First, the PrPC increase itself appears too great (up to 100-fold) to be explained by a stabilization of plasma PrP, especially as protein–antibody complexes are usually a better target for catabolism in the circulating compartment (Lobo et al., 2004).
- Second, in tga20 mice, which overexpress PrPC by about 10-fold, PrPC–antibody complexes appear rapidly and abundantly in plasma, but their presence is only transient. We did not observe particularly high clearance of free antibodies (which do not bind to PrPC), which implies a low stability of PrPC–antibody complexes in plasma in these mice.
- Finally, there is a similar increase in plasma of PrP fragments, which do not bind to antibodies used for treatment, thus ruling out greater stability of PrPC when complexed with antibodies as the major cause of the large increase in plasma PrPC.

One hypothesis is that massive release of PrPC from a blood compartment (white blood cells) or from peripheral lymphoid tissues into plasma could be associated with a depletion of PrPC in these cells/tissues, with consequent impairment of peripheral PrPSc neosynthesis. An alternative hypothesis is a direct interaction between antibodies and PrPSc or a direct therapeutic activity of PrPC–

![Fig. 5. Characterization of plasma PrPC in tga20 mice. tga20 mice were injected i.p. with 1 mg Pri308, βS23 or BAR236 antibody and bled before injection (lanes 1 and 3) and 6 h after injection (lanes 2 and 4). Plasma PrPC was first immunoprecipitated with magnetic beads coupled to either mAb Sha31 (lanes 1 and 2) or mAb SAF34 (lanes 3 and 4) (see Methods). Immunoprecipitated PrPC was detected by Western blotting with either Sha31 coupled to HRP (left membranes) or biotinylated SAF32 and streptavidin coupled to HRP (right membranes).](image-url)
antibody complexes on the transconformation process, reminiscent of a PrP-FC$_2$-like molecule (Meier et al., 2003).

We are currently testing these hypotheses to identify the tissues and cell compartments that could be the source of PrPC released into plasma and to evaluate the therapeutic effect of PrPC-antibody complexes. We may also repeat some of the experiments using Fab’ and Fab’$_2$ fragments (modified via pegylation (Fishburn, 2008) or not) of some of the mAbs to understand better the underlying mechanisms.

**METHODS**

**mAbs.** mAbs are described in Table 1. Epitope mapping to identify linear epitopes was performed as described previously (Morel et al., 2004). All mAbs were used for passive immunization as dialysed and filtered ascitic fluid. As the concentration of mAb in the ascites varies greatly between hybridomas, all ascitic fluids were titrated before diluting accordingly in PBS to reach a final concentration of 5 mg ml$^{-1}$. Titration was performed by ELISA using dilutions of each affinity-purified mAb to set up standard curves. Ascitic fluids may also contain trace amounts of endogenous mouse antibodies.

**Antibody pharmacodynamics**

**Mouse lines.** Three mouse lines, C57BL/6 wild-type (Janvier), tga20 and Prnp$^{E95}$ transgenic (with 12595 × C57BL/6 genetic background; CDTA) mice, were used, involving mice between 7 and 9 weeks old.

**Experimental challenge and treatment.** We first investigated the efficacy of short and early mAb treatment in C57BL/6 (n = 7) and tga20 (n = 6; overexpressing PrPC by about 10-fold (Fischer et al., 1992, 1993), (ii) normal PrPC levels (C57BL/6) and (iii) 10-fold-higher PrPC levels (tga20). Blood (around 50 µl) was sampled from each mouse (n = 3 for each line) before treatment and 6 h, 2, 4, 7, 10, 14 and 21 days post-treatment (single i.p. injection with 1 mg mAb in 500 µl volume). Controls consisted of injections of 500 µl PBS. EDTA blood samples were centrifuged (5 min, 3000 g) and collected plasma was stored at −20 °C.

**ELISA free mAb measurement.** Mouse plasma was used diluted 1:100 in EIA buffer [0.1 M phosphate buffer (pH 7.4), 0.1% BSA, 0.15 M NaCl, 0.01% sodium azide]. Serial dilutions of 50 µl plasma mixed with 50 µl PrP/peptide tracer were reacted at 4 °C overnight in a 96-well microplate (Maxisorb immunoplate; Nunc) coated with goat anti-mouse IgG antibody (10 µg ml$^{-1}$; Jackson ImmunoResearch). Serial dilutions of corresponding purified and titrated mAb were used as standard. PrP/peptide tracer coverage to peptide Pri3 (106-KTNMKHMAGAAAAGAVGGLG-126) coupled covalently to acetylcholinesterase enzyme (Pri3–AChE, used at 2 U ml$^{-1}$) for Pri308 titration, peptide SP-Cyst(3)--AChE used at 2 U ml$^{-1}$ for NSP35-6, 20 ng ml$^{-1}$ of biotinylated N-terminal amyloid peptide for AMYL-2, 20 ng ml$^{-1}$ of biotinylated peptide PriRep1 (79-GGWGQPHEGWWGGQG-92) for SAF34, 20 ng ml$^{-1}$ of biotinylated peptide Pri4D (142-GSDYEDRYRENM-HRPQ5-160) for Sha31, or 50 ng ml$^{-1}$ of biotinylated recombinant x-folded murine PrP (from C. M. Sorgato, University of Padova, Italy) for all other mAbs. After three washes, plates containing a biotinylated tracer reacted for 1 h at room temperature with 100 µl per well of 2 U ml$^{-1}$ of AChE-labelled streptavidin. After six washes, AChE activity was revealed by Ellman’s colorimetric method (Grassi et al., 1989) at 414 nm after 30 min.

**ELISA of PrPC in plasma.** Serial dilutions of plasma (100 µl) were reacted overnight at 4 °C in 11C6 anti-PrP mAb-coated (10 µg ml$^{-1}$) 96-well microplates. After three washes, plates were reacted for 2 h at room temperature with 100 µl per well of a SAF83–AChE tracer antibody (5 U ml$^{-1}$), before washing and revealing. A recombinant x-folded murine PrP was used as standard.

This sandwich immunoassay can measure both free PrPC and some of the PrPC-antibody complexes. However, the presence of the antibodies used to treat mice can interfere with this sandwich immunoassay, as observed clearly in synthetic samples composed of plasma spiked with known amounts of antibodies. Almost all of the mAbs, except for the control antibody Pri308, spuriously reduced the sandwiching of PrPC in a dose-dependent manner (mAb concentration range, 20–350 µg ml$^{-1}$). As expected, this interference was particularly important with 11C6 and SAF83, but also with BAR236 and Sha31, leading to important underestimation of plasma PrPC [from ~60% (11C6) to ~100% (Sha31) when using 100 µg ml$^{-1}$]. The interference was less important with SAF34, J523 and BAR221 (from 45 to 20%). In conclusion, total plasma PrPC concentrations measured by our sandwich immunoassay were underevaluated, as part of the complexed PrPC was ignored.
In order to circumvent this problem, in some plasma samples, bound and free fractions of PrP were separated by precipitation of immunoglobulins, performed by mixing 500 µl of 1:100-diluted plasma with 25 µl protein A-Sepharose 4B (fast flow; Sigma) for 6 h at room temperature. Supernatant containing free PrP was collected, whilst PrP in the protein A-bound fraction (PrP from complexes) was removed from the gel (after washing) by heating at 100 °C for 5 min in 25 µl denaturing buffer C1 (from the Bio-Rad TeSE® Sheep and Goat kit).

Measurement of PrP–antibody complexes by solid-phase immobilized epitope immunoassay (SPIE-IA). SPIE-IA (Grassi et al., 1996; Volland et al., 1999) was used to quantify mAb–PrP complexes in plasma. One hundred microlitres of 1:100-diluted mAb–PrP complexes in plasma. One hundred microlitres of 1:100-diluted antibody complexes were cross-linked by using 0.1 % glutaraldehyde in phosphate buffer, pH 7.4 (5 min at room temperature), before washing. Residual glutaraldehyde reactivity was neutralized with 100 µl borane trimethylamine (10 mg ml⁻¹; Sigma) in methanol/HC1 per well, and solid-phase bound complexes were denatured by 100 µl 1 M NaOH (5 min at room temperature). Solid-phase mAb–PrP complexes were detected by reacting plates for 2 h at room temperature with 100 µl SAF38–AChE tracer per well (recognizing a discontinuous epitope in the central region of PrP; Table 1), prepared in EIA buffer containing 10 % murine Erlich ascitic fluid (saturation). AChE activity was measured by the colorimetric method of Ellman (Grassi et al., 2000) after 30 min.

Characterization of PrP in plasma. N-terminal processing of plasma PrP in C57BL/6 mice was characterized biochemically by sandwich immunoassays (Fig. 4) using the same procedure as described above:

(i) 11C6/SAF83–AChE sandwiching to detect both full-length and N-terminally truncated PrP,
(ii) 11C6/SAF34–AChE sandwiching to detect only full-length PrP, and
(iii) SPIE-IA (Volland et al., 1999) with a SAF34/SAF34–AChE format to detect specifically N-terminal fragments after removal of full-length and N-terminally truncated PrP by Sha31 immunoprecipitation (Morel et al., 2004).

N-terminal processing of plasma PrP in tga20 mice was characterized biochemically by immunoblotting (Fig. 5). First, 50 µl plasma was diluted 10-fold in 0.1 M phosphate buffer (pH 7.4). Then, 10 µl (2 × 10⁶ beads) tosylated Dynabeads M-280 (Dynal) coupled to either mAb Sha31 or mAb SAF34 was added and reacted overnight at 4 °C with rotation. Beads were then washed twice in 0.1 M phosphate buffer, 1 % Tween 20, and once in 0.1 M phosphate buffer without Tween 20, before heating to 100 °C for 5 min in 15 µl loading buffer without reducing agent. Samples eluted from beads were run on SDS-PAGE (12 % resolving). Proteins were blotted onto PVDF membranes (Amersham) blocked with 2 % BSA. Immunoprecipitated PrP was detected either with Sha31 coupled to horseradish peroxidase (HRP) or biotinylated SAF32 (antibody recognizing the octarepeat region of PrP) and streptavidin coupled to HRP, further revealed by chemiluminescence (ECL plus; Amersham).

Pharmacokinetic analysis. A non-compartmental and statistical moment model was applied for the determination of pharmacokinetic parameters of mAbs, mAb–PrP complexes and free PrP concentrations (Kinetica version 3.0; Inaphase). This non-compartmental model was chosen because of the reduced number of time points available in our pharmacokinetic study. The maximum concentration (Cmax) and time to maximum concentration (Tmax) were determined directly from plasma concentrations, whilst all the other parameters [mean residence time, apparent steady-state volume of distribution (Vss), clearance, half-life of elimination (t1/2)] were calculated by using the log-linear trapezoidal rule with extrapolation to infinity.

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REFERENCES

(PrP) with amino-proximal deletions restoring susceptibility of PrP knockout mice to scrapie. *EMBO J* 15, 1255–1264.


