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Reduced Immune Detection of the Pathogenic Prion Protein in Presence of Triose Phosphate Isomerase

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During performing electrophoresis for detecting the presence of recombinant prion protein (PrP^{rec}), it was punctually planned to examine several expressed protein on the same polyacrylamide gel and the last empty well was used for depositing together the molecular weight marker (triose phosphate isomerase) and the pathogenic bovine prion protein PrP^{sc}. After transfer and immune-blotting with specific PrP monoclonal antibodies [1] no detectable PrP^{sc} bands were observed. So we decided to explore these unexpected results.

The -helix rich native host cellular encoded glycoprotein, designated (PrPc), is a widely occurring protein, whose sequence is well conserved in mammals. Conformational changes in PrPc lead to propagation of the pathogenic protein PrP^{sc} which is a β-sheet aggregates, detergent insoluble and resistant to Proteinase K (PrPres) [1-3]. The accumulation of the PrP^{sc} in animals and man is associated with a wide range of transmissible neurodegenerative spongiform encephalopathies; including scrapie in sheep, spongiform encephalopathy in cattle (BSE), chronic wasting disease in deer and Creuztfeldt-Jacob disease in humans. This group of disorders is also called prion diseases because they are caused by an infectious protein (PrP^{sc}).

Triose phosphate isomerase (TPI) a glycoprotein formed by a dimer of identical subunits, each of which is made up of about 250 amino acid residues with a molecular weight of 28 KD and is the key enzyme in cell metabolism controlling the glycolytic flow and energy production. The functional deficiency in TPI glycolytic enzyme activity is associated to neurodegeneration. In particular, inefficient glycolysis and ATP depletion which are also characteristic in Alzheimer's disease brains.

Alzheimer's disease, as other amyloidosis, presents insoluble accumulations of β-sheet containing proteins. A β-induced oxidative and nitrative stress and nitrotyrosination of TPI causing the generation of toxic intermediates and abnormal glycation of proteins, as well as a conformational change in and aggregation of the TPI itself. The large size of nitrotyronisated TPI aggregates make them resistant to degradation by the proteasome and can then grow and act as intracellular 'seeds' for the fibrillation of other proteins. More interestingly, the nitrotyronisated TPI is able to induce a conformational change in tau leading to precipitation of abundant paired helical filament like formation similar with the twisted fibrils structure that is also seen in Alzheimer's disease brains [4].

The TPI was obtained from Sigma (ref. T 9400 and T 5806). The vial content 5 mg was dissolved in 2 ml Laemmli denaturing buffer, heated for 3 min. at 100°C, vortexed, reheated once more and vortexed then dispensed in small volumes and kept at -20°C.

Due to The appearance of turbidity after adding a constant or variable dilutions of TPI to different volumes of proteinase K treated pathogenic prion protein (PrP^{res}), the mixtures were vortexed, heated for 5 min. at 100°C and centrifuged for two min. at 10000 RPM. The supernatants were collected and a constant volume from each was deposited and run on 15% SDS acrylamide gel, transfer on nitrocellose membrane and immunoblotted using either an anti-PrP immune rabbit serum (RB1) or monoclonal antibodies directed to different zones of the PrP peptide sequence and peroxidase conjugated anti-rabbit or anti-mouse polyclonal serum (Figure 1).

The results in Figure 2 were obtained after ½ diluting 6 µl of bovine PrP^{res} in an equal volumes (6 µl) of Laemmli buffer containing either 0, 6, 3 or 1.5 µl TPI respectively.

The results present in Figure 3 were obtained by adding in double, 0, 2 or 4 µL TPI and 4, 2 or 0 µl buffer to constant volume (2 µl) of PNGase partially treated (**A**) or (1 µl) of untreated (**B**) Sheep PrP^{sc}, showed again a decreased bands intensity in parallel with the TPI volume added. Note a white zone (pointer) represent the position of the TPI band in the gel.

The results in Figure 4 were observed after electrophoresis of the supernatant obtained after vortexing, heating and centrifugation of mixtures from two sets; one containing only either 1.2, 0.8, 0.4 or 0.2 µl of bovine PrPres alone and the other set contained beside the PrPres 2 µl TPI and the final volume of each tube of both sets was brought to 5.2 µl with Laemmli buffer. Electrophoresis was done on 15% SDS acrylamide gel, transfer on nitrocellose membrane and a double immunoblotted using first a monoclonal anti-PrP^{sc} antibody then a peroxidase conjugated anti-mouse polyclonal serum followed by washing in PBS overnight at laboratory temperature. The second immunoblotting with done using a rabbit polyclonal anti PrP^{sc} serum then a peroxidase conjugated anti-rabbit serum.

The results in Figure 5 were observed after adding in two tubes out of four composing each of the 4 prepared sets was added 16 µl and 8 µl of mice PrPres to the 2 other tubes. To only one of the two tubes containing either 16 or 8 µl was added 16 µl of TPI. Buffer was added to adjust the final volume of each tube to 32 µl, the mixtures were vortexed, heated for 5 min. at 100°C and centrifuged for two min. at 10000 RPM. The supernatants were collected and a constant volume from each was deposited and run on 15% SDS acrylamide gel, transfer on nitrocellose membrane and immunoblotted using either one of 4 monoclonal antibodies. The results in figure 5 were observed .....monoclonal antibodies. The epitope for Pri 917 is at 214-230 aa, for 8G8 at 95-110 aa, for 3B5 at 79-92 aa and for Saf 70 is at 142-160 aa.

To confirm that the PrP^{sc} reduced bands intensity was probably due to destruction of the PrP^{sc} infectivity serial dilutions from 10^{-1} to 10^{-7} were prepared using a 2% brain suspension of the mouse adapted strain

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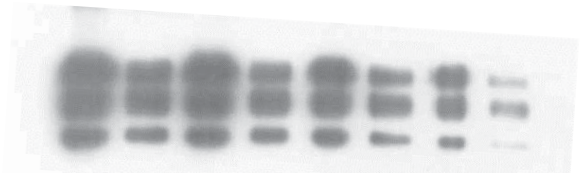


PrP^{sc}	4	4	3	3	2	2	1	1 µl
Buffer	4	0	5	1	6	2	7	3 µl
TPI	0	4	0	4	0	4	0	4 µl

Figure 1: Two sets of 4, 3, 2 and 1 µl of mice PrPsc were prepared and to the first set was added 4, 5, 6 or 7 µl buffer and to the second set 4 µl TPI and completed by 0, 1, 2 or 3 µl buffer respectively. After electrophoresis and immunoblotting a decreased immune detectability of the prion protein bands in presence of TPI was observed compared to controls.

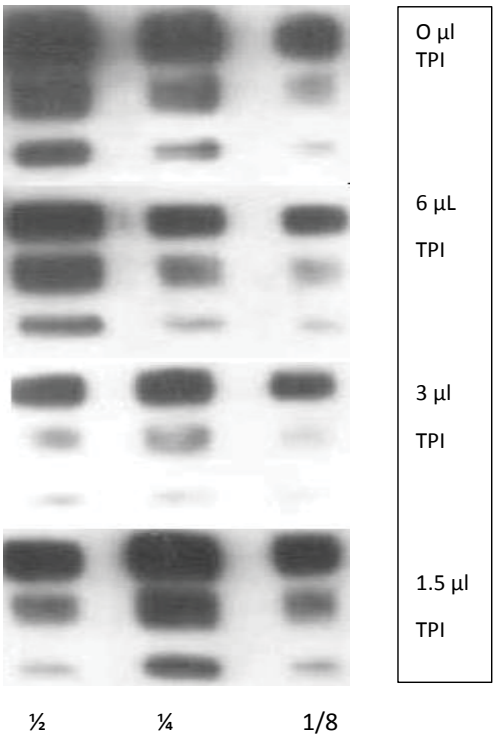


Figure 2: The comparison of the Pr^{Pres} bands intensity in absence and in presence of different volumes of TPI showed a zone phenomenon. The lowest immune detectability was in presence of 3 µl TPI then in presence of 1.5 µl TPI the bands intensity increased.

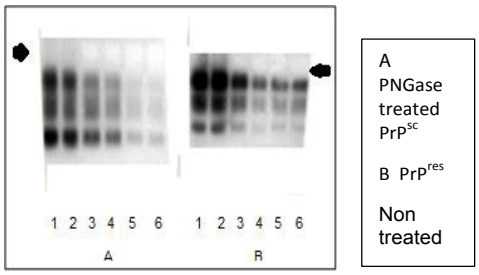
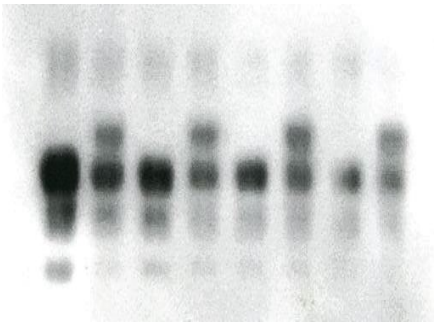


Figure 3: Reduced immune bands detectability of PNGase partially treated and untreated sheep PrP^{sc} in presence of 0, 2 or 4 µl TPI respectively. Pointer indicates a white zone representing the position of the TPI band.



	1	2	3	4	5	6	7	8
PrPsc	1.2	1.2	0.8	0.8	0.4	0.4	0.2	0.2 µl
TPI	0	2	0	2	0	2	0	2 µl
Buf	4	2	4.4	2.4	4.8	2.8	5	3 µl

Figure 4: In presence of TPI the PrP bands showed a reduced immune detectability compared to controls. Also in presence of TPI but not in the controls an unexpected immune detected new band having a molecular weight of 33 KD was observed just above the PrPsc upper bi-glycosylated 29 KD band.

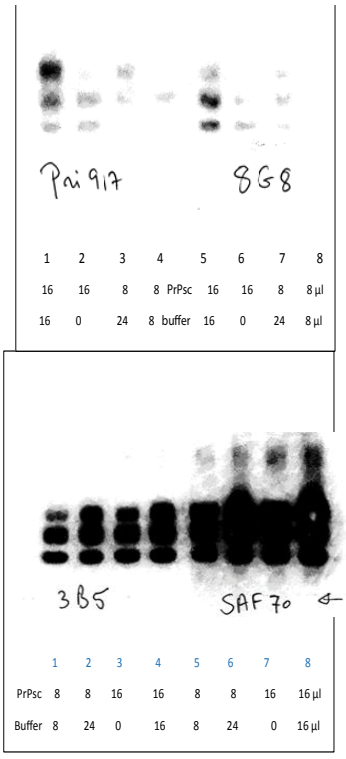


Figure 5: The immune staining with each of the monoclonal antibodies revealed a decreased reactivity in presence of higher TPI content. Also difference in the reactivity of each monoclonal with the mice PrPres were previously reported.

C506M3 in 5% glucose saline buffer. Each dilution from 10^{-2} to 10^{-7} was divided into 3 sets each 300 µl volume. The dilutions 10^{-2} to 10^{-7} alone represent the control, to each tube of the second set from 10^{-4} to 10^{-7} (**A**) was added 1 µl TPI and to each tube from 10^{-3} to 10^{-7} representing the third set (B) was added 6 µl TPI.

Ten C57BL/6 mice were inoculated with 20 µl volume intracranially with every dilution from each set. The mice were controlled

daily for developing symptoms or death and brains of diseased or did animals were collected and kept at -20°C tell the terminal stage of the study (63 days). The brains were individually homogenized at 10% (w/v) in 5% glucose solution. The homogenates were incubated in presence of 80-100 µg/ml proteinase K for one hour at 37°C. After addition of Laemmli denaturation buffer, 5 min. heating at 100°C, 5 min. centrifugation at 10 000 RPM the supernatants were collected and deposited on 15% SDS-Polyacrylamide gel electrophoresis, following transfer to nitrocellose membranes, the presence of PrP^{sc} were detected using SAF84 anti-PrP mABs and chemiluminescence detection system (Moussa) (Tables 1 and 2).

The results obtained showed that there is a complete infectivity reduction at the dilution of 10^{-4} in presence of 1 µl TPI and variable infectivity reduction in presence of 6 µl TPI at the dilutions 10-3 and 10^{-4}. Due to the presence of turbidity in mixtures of TPI and PrP^{sc}, the measurement of absorbance at 300 nm in ultraviolet spectroscopy was not possible to reveal the interaction between TPI and PrP^{sc}. All the results were obtained by using only the supernatants collected after the mixtures of TPI and PrPres were vortexed, heated for 5 min at 100°C and centrifuged for two min. at 10000 RPM. The precipitates present after centrifugation was not examined after suspension in electrophoresis. The addition of TPI to PrP^{sc} from either one of the different animal species induced always a reduction in the immune detectability of the prion protein.

The presence of a new unsuspected PrP immune detected protein band at 33 KD position was not revealed earlier during the previous immuno-blotting experiments using only one antibody. This band represent the interaction between the TPI and the constituent of the PrPres and was revealed only after immune detection using a monoclonal antibody, washing with PBS then incubation with a peroxidase conjugated anti-mouse polyclonal serum. Rewashing with PBS buffer overnight and re-immune detection by the anti-PrP^{sc} rabbit hyper immune serum, washing and finally incubation with a peroxidase conjugated anti-rabbit polyclonal serum. This interaction can be assimilated to that occurring between TPI and tau protein *in vitro* and seen in brains of Alzheimer disease [4]. Finally, infectivity reduction was detected after inoculation of mice with mixture of PrP^{sc} and TPI compared to the controls.

	Control	1 µl TPI	6 µl TPI
Dilution	**D/A/C**	**D/A/C**	**D/A/C**
10^{-2}	08/00/02	NT	NT
10^{-3}	06/03/01	NT	01/06/03
10^{-4}	04/05/01	01/08/01	03/07/00
10^{-5}	01/08/01	07/03/00	02/08/00
10^{-6}	01/09/00	00/09/00	00/09/01
10^{-7}	00/10/00	00/09/01	00/09/01

Table 1: Final observation results: Dead (D), Alive (A) and Cannibalized (C) and not tested (NT).

Dilution	Control	1 µl TPI	6 µl TPI
10^{-2}	8/8	NT	NT
10^{-3}	6/9	NT	1/7
10^{-4}	4/9	0/8	3/10
10^{-5}	0/9	0/10	0/10
10^{-6}	0/10	0/9	0/9
10^{-7}	0/9	0/9	0/9

Table 2: Westrn-blot results: PrP^{sc} positive mice brains/total examined.

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